Novel insights into MGL-glycan interactions in the immune system

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Sandra Johanna van Vliet

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Novel insights into MGL-glycan interactions in the immune system

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Sandra Johanna van Vliet

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promotor: prof.dr. Y. van Kooyk

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

Marie Curie

voor mama
voor papa

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CONTENTS

ABBREVIATIONS

AaMϕ	Alternatively activated macrophage
APC	Antigen presenting cell
ASGP-R	Asialoglycoprotein receptor
CARD	Caspase recruitment domain
CHO cells	Chinese hamster ovary cells
CLR	C-type lectin receptor
CR	Cysteine-rich domain
CRD	Carbohydrate recognition domain
CSF-1	Colony-stimulating factor-1
CTL	Cytotoxic T cell
CTLD	C-type lectin-like domain
DC	Dendritic cell
DCIR	Dendritic cell immunoreceptor
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing nonintegrin
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic Reticulum
FNII	Fibronectin type II domain
GalNAc	N-acetylgalactosamine
GBS	Guillain-Barré syndrome
GC	Glucocorticoids
GILZ	Glucocorticoid-induced leucine zipper
GlcNAc	N-acetylglucosamine
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
hMGL	Human MGL
HPA	*Helix pomatia* agglutinin
HUVEC	Human umbilical vein endothelial cells
IgAN	IgA nephropathy
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LC	Langerhans cell
LDN	LacdiNAc or GalNacβ1-4GlcNAcβ
LDNF	Fucosylated LacdiNAc or GalNacβ1-4(Fucα1-3)GlcNAcβ
Le^X	Lewis X or Galβ1-4(Fucα1-3)GlcNAc
LN	Lymph node
LNFP III	Lacto-N-fucopentaose III

LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
L-SIGN	Liver/Lymph node-specific ICAM-3-grabbing nonintegrin
MAA	*Maackia amurensis* agglutinin
mAb	Monoclonal antibody
M-ASGP-BP	Macrophage asialoglycoprotein-binding protein
MFI	Mean fluorescent intensity
MGL	Macrophage galactose-type lectin
MMP-13	Matrix metalloprotease-13
Mϕ	Macrophage
MoDC	Monocyte derived dendritic cell
MR	Mannose Receptor
NO	Nitric oxide
OVA	Ovalbumin
PAA	Polyacrylamide
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
pgl	Protein glycosylation cluster (*Campylobacter jejuni*)
PPR	Pattern recognition receptor
PLA_2R	Phosholipase A_2 receptor
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SARS	Severe acute respiratory syndrome coronavirus
SBA	Soybean agglutinin
SEA	Soluble egg antigens (*Schistosoma mansoni*)
SNA	*Sambucus nigra* agglutinin
TCR	T cell receptor
T_{eff}	Effector T cells
TF antigen	Thomsen-Friedenreich antigen
Th	T helper
TLR	Toll-like receptor
T_{reg}	Regulatory T cell
UEA-1	*Ulex europaeus* agglutinin-1
uPAR	Urokinase plasminogen activator receptor

CHAPTER 1

GENERAL INTRODUCTION

DENDRITIC CELL AND MACROPHAGE IMMUNOBIOLOGY

Antigen presenting cells

In 1882 the Russian embryologist Ilya Mechnikov stuck the thorn of a tangerine tree in his laboratory animal, a starfish larva, and observed how a population of mobile cells attacked the inserted splinter. Based on this experiment he established his phagocytosis theory in 1883, an active host reponse to pathogens[1]. The current view held at that time stated that pathogens were expelled by depriving them of nutrients. In contrast, Mechnikov proposed that phagocytes actively protected the host and recognized nonself in every form, from pathogenic to malignant or damaged tissue. He named the cells that played a role in host defense by phagocytosis microphages (now better known as polymorphonuclear leukocytes) and mononuclear macrophages. For his revolutionary concept of innate immunity, which is still valid today, he was awarded the Nobel Prize for Medicine in 1908.

Although macrophages are now recognized as a central player in innate and adaptive immunity, dendritic cells (DCs) are by far the most potent antigen presenting cell (APC) in the immune system for driving adaptive T cell responses. In 1868 Paul Langerhans had already described a DC subpopulation in human skin, the Langerhans cell (LC), but he mistakenly classified them as nerve cells[2]. Not until 1973, the function of DCs became clear when Ralph Steinman and Zanvil Cohn reported a novel cell type in murine lymphoid organs, which they named dendritic cells, after their many unique cytoplasmic extensions or dendrites[3]. These DCs proved to be 100 fold more effective at stimulating spleen cells in primary allogeneic mixed leukocyte reactions than lymphocytes or macrophages[4].

Although mostly regarded as completely separate leukocyte lineages, recent evidence indicates that DCs and macrophages renew from a common bone marrow progenitor that is able to develop into both subtypes depending on differential cytokine signaling[5]. Intriguingly, mice lacking the receptor for the key growth factor for macrophage development, colony-stimulating factor-1 (CSF-1), are also completely devoid of LCs, suggesting a combined regulatory network for the development of both DCs and macrophages[6]. Furthermore, all murine spleen plasmacytoid and $CD11c^+$ DC subsets express the CSF-1 receptor during differentiation and their cell numbers are equally dependent on the presence of CSF-1[7]. These data indicate that there is no clear distinction between DCs and other phagocytes, in contrast a continuum exists of cellular phenotypes that may be defined at the extremes as classically activated macrophages and mature DCs[8].

Pattern recognition

To perform their unique bridging function between innate and adaptive immunity DCs and macrophages are located at strategic sites in the body, namely throughout all peripheral tissues, where they act as sentinels, scanning their surroundings for incoming pathogens or local environmental changes. In order to correctly decipher environmental clues, these APCs are equipped with a vast array of pattern

recognition receptors (PPRs), germ-line encoded proteins that specifically recognize signature molecules from pathogens or damaged tissues. Toll-like receptors (TLRs) interact with pathogen-associated molecular patterns (PAMPs), microbial structures from bacterial, viral, fungal or parasitic origin[9]. In addition, they bind a variety of self-proteins including several heat-shock proteins. Some TLRs (1, 2, 4, 5 and 6) are found on the cell surface, whereas other TLRs (3, 7, 8, and 9) reside within the endosomal compartment. Besides the TLRs, APCs express cytoplasmic PPRs belonging to the NOD-like family and the RIG-like family, which recognize bacteria and viruses, respectively[10]. Moreover, C-type lectins and scavenger receptors can also participate as pathogen sensors and thus function as PPRs[11]. The extensive range of PPRs, chemokine/cytokine receptors and adhesion molecules provides APCs with the unique ability to adapt to local conditions, via endogenous or exogenous signals, and adopt several phenotypic profiles or activation states (Fig. 1 and 2).

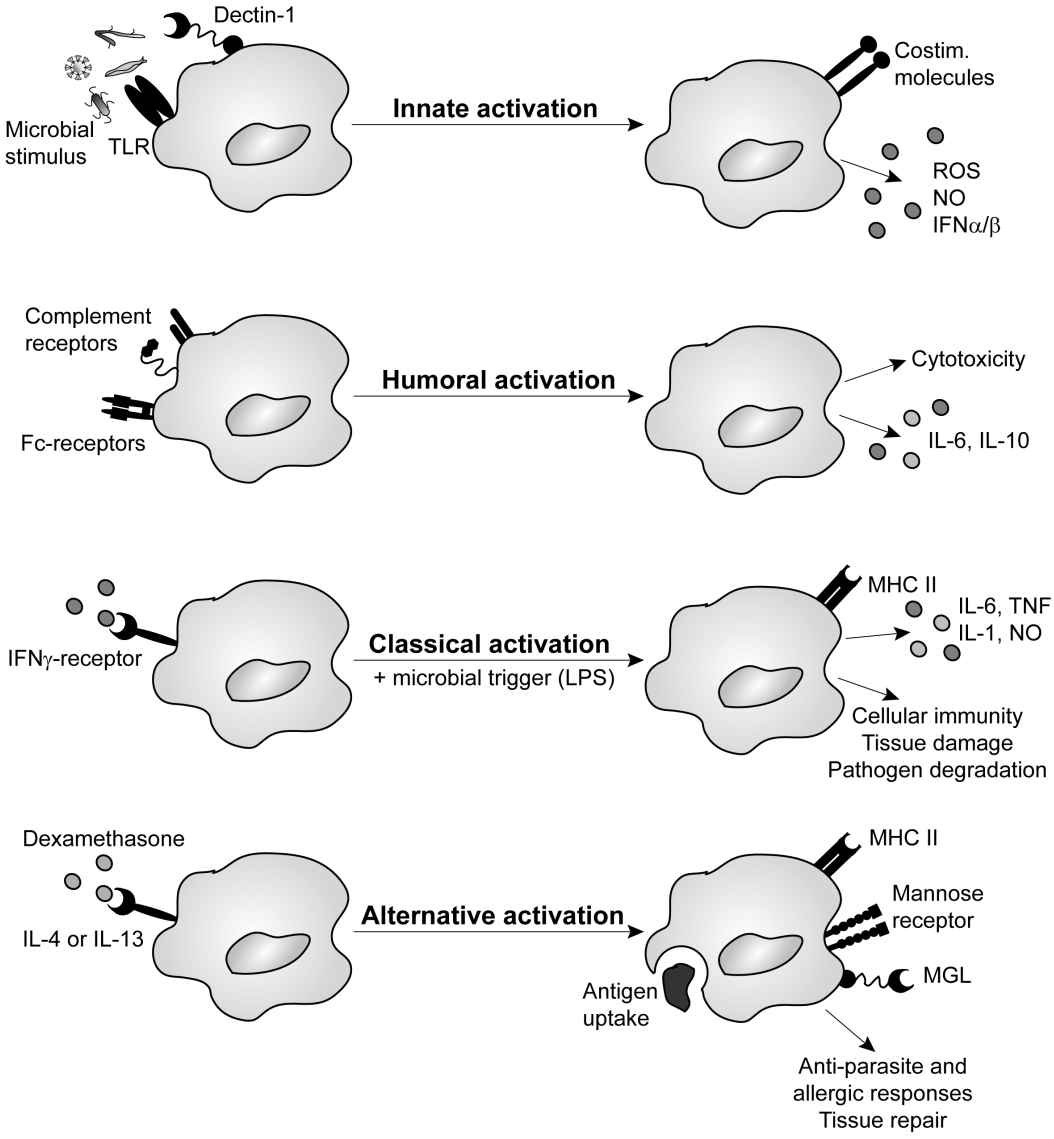

Figure 1. Functional polarization of macrophages. Adapted from Gordon[12].

Functional specialization of macrophages

Macrophages have long been characterized on the basis of morphology, expression of enzymes, like acid phosphatase and non-specific uptake of inert particles[13]. This diverse group of phagocytes with very specific "members" in different tissues, ranging from the Kupffer cells in the liver, alveolar macrophages, placental Hofbauer cells, to microglial cells in the brain, likely reflects a functional versatility of these cells in response to different environmental signals.

Innate activation of macrophages following pathogen binding to TLRs or the C-type lectin Dectin-1, results in secretion of proinflammatory cytokines, reactive oxygen species and the upregulation of costimulatory molecules[14]. If the microbial stimulus is combined with IFNγ exposure, macrophages adopt the classically activated phenotype, characterized by high production of IL-12 and inflammatory mediators such as nitric oxide (NO). These classically activated macrophages are particularly capable of killing intracellular pathogens and they promote Th1 immunity. Nonetheless, classically activated macrophages must be kept under tight control, as an excessive amount of their secretion products can cause extensive tissue damage[15]. In contrast, when immature macrophages are under the influence of IL-4 or glucocorticoids, they differentiate into alternatively activated macrophages. These alternatively activated macrophages do not produce IL-12 or NO; instead they upregulate receptors for antigen uptake, such as the mannose receptor (MR) and macrophage galactose-type lectin (MGL)[12,16]. Although they are poor stimulators of T cell proliferation, they promote tissue repair and wound healing through the synthesis of extracellular matrix components[17,18]. Alternatively activated macrophages actively contribute to the immune response against helminth parasites[19]. Type II or humoral activation of macrophages by immune complexes induces secretion IL-10 and promotes Th2 immunity[12,15]. Macrophages have also been categorized according to their cytokine profiles, M1 macrophages are typically IL-12^{high} and IL-10^{low} (corresponding to the classically activated phenotype), whereas M2 macrophages are characterized as IL-12^{low} and IL-10^{high} (alternatively activated and humoral phenotype)[20].

Although commonly regarded as tissue-resident cells, recent data indicate that macrophages are able to migrate towards the lymph node and prime naive CD8$^+$ T cells, however with lower efficiencies than DCs[21].

Dendritic cells, maturation and tolerance

In humans several pathways exist for the development of different DC subtypes, such as myeloid DCs, LCs, plamacytoid DCs and interstitial DCs, each requiring their own set of growth factors and/or cytokines. Therefore, different DC lineages may develop from separate precursors or represent various activation states of a single subtype[22]. Resident DCs in peripheral tissues are termed immature to define a stage of environmental monitoring and antigen sampling before DC activation occurs. These immature DCs generally have a high endocytic capacity, however to become fully immunogenic an essential maturation stimulus is required that can be

achieved by TLR triggering or by licensing via innate immune cells through pro-inflammatory cytokines and CD40-CD40ligand interactions[23-26]. DC maturation is followed by a transient wave of enhanced antigen uptake; only after terminal maturation the endocytic capacity of DCs is shutdown[27]. Endocytosed antigens are subsequently degraded for presentation onto MHC class II. A specialized feature of DCs is their ability to load exogeneous ligands into the MHC class I pathway, a process termed cross-presentation[28]. Priming of *de novo* T cell responses occurs in the draining lymph nodes, so maturing DCs migrate from the peripheral tissues to the lymph nodes according to the upregulated expression of the chemokine receptor CCR7[29]. Three signals determine the fate of the interacting naive T cells, the strength

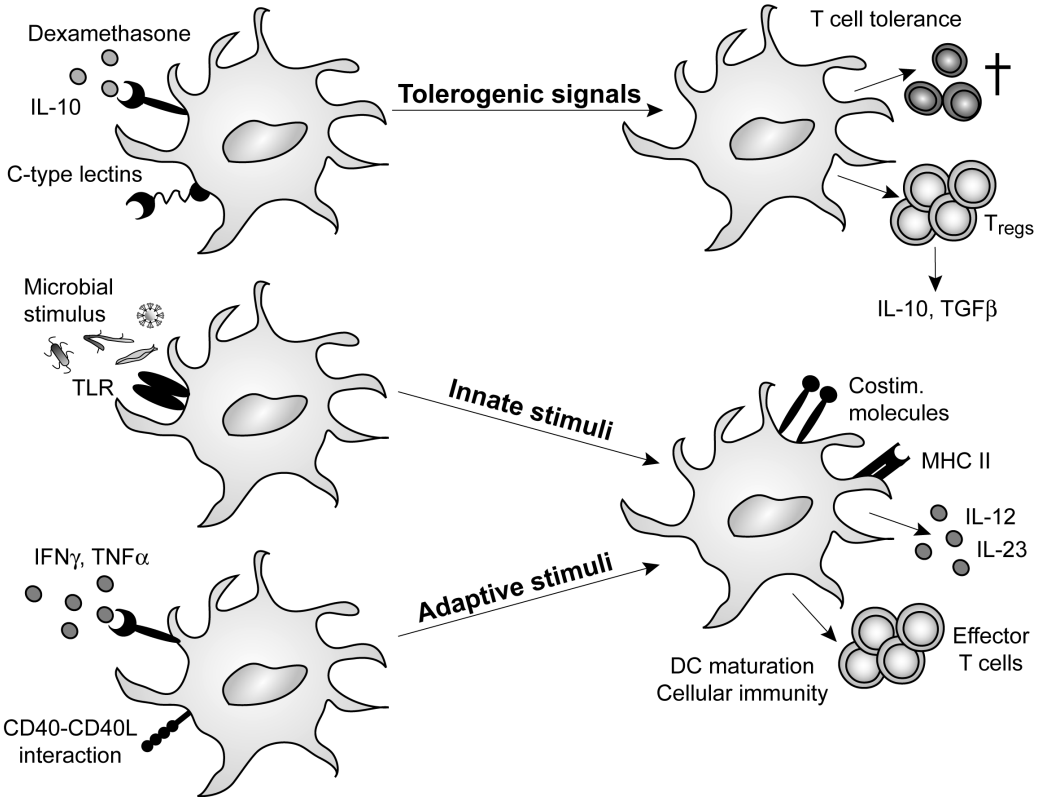

Figure 2. Functional polarization of DC.

of the peptide/MHC-T cell receptor interaction, the amount of costimulation and the functional polarization by DC expressed cell surface molecules or DC-derived cytokines[30]. High IL-12 production by DCs drives the differentiation of Th1 effector T cells, important for cellular immunity and eradication of intracellular pathogens. Conversely, IL-4 promotes the development of Th2 cells that contribute to immunity to parasites and in allergic reactions. Although IL-4 is the key factor in driving Th2 polarization, other lymphocytes and not DCs seem to be responsible for the IL-4 production[31]. Recently, IL-23 production by DCs was shown to control the development of a new $CD4^+$ Th subset, the Th17 cells, named after their high production of IL-17[32]. These cells are critical in the host defense against extracellular

bacteria, such as *Klebsiella pneumoniae*, however they are also implicated in the onset of several autoimmune diseases[33,34].
Although initiation of adaptive immunity is the hallmark of DC function, DCs also actively induce and maintain tolerance in the steady state. Even under steady state conditions some migration of semi-mature DCs to the lymphatics can be observed[35]. These semi-mature DCs can present self-antigens to T cells, however T cell immunity is not induced. Instead these tolerogenic DCs instruct regulatory or suppressor T cells, cause T cell anergy or hyporesponsiveness, or delete the responder T cells from the repertoire[36,37]. As negative selection in the thymus is insufficient to eliminate all autoreactive T cells from the repertoire, these tolerogenic DCs probably function to limit autoimmunity[38]. IL-10 and substances such as glucocorticoids also instruct the formation of such tolerogenic DCs[39-41]. Moreover, targeting of antigens without overt danger signals to certain C-type lectins on DCs is followed by subsequent deletion of responder T cells[42].
Thus, the heterogeneity and plasticity of APCs is closely related to the multitude of immunological functions performed by DCs and macrophages (Fig. 1 and 2). Recently, many novel cell surface molecules have been identified that are involved in the orchestration of both immunity and tolerance by APCs. In particular, the large diversity of APC-expressed C-type lectins has been implicated not only in pathogen recognition, but also in the functional modulation of the DC or macrophage itself[43].

C-TYPE LECTIN AND C-TYPE LECTIN-LIKE MOLECULES

Structure and carbohydrate recognition

In the past, glycosylation (see appendix) was often regarded as a mere decoration of proteins. Yet all organisms contain specialized lectin receptors for recognizing carbohydrate structures present on cell surfaces, attached to circulating proteins or in extracellular matrices. By binding to the carbohydrate moieties, lectins specifically mediate biological events, such as cell-cell or cell-matrix adhesion, host-pathogen recognition, serum glycoprotein turnover, and innate immune responses. Lectin classification is based on the primary protein sequence of the carbohydrate recognition domain (CRD), the protein domain involved in carbohydrate binding. One of the most extensively studied lectin families are the C-type lectins. Hallmark of classical C-type lectins is the dependence on Ca^{2+} for carbohydrate recognition. Loss of the Ca^{2+}-ion, which is coordinated by the primary binding site within the CRD, results in conformational changes and loss of binding function[44]. This primary binding site is selective for single monosaccharide residues. A secondary binding site exists adjacent to the primary site that interacts with neighboring monosaccharide residues present in the interacting carbohydrate structure. This secondary binding site strongly enhances binding affinity and fine-tunes lectin specificity for certain types of linkages and substitutions[45]. Many other proteins contain large parts of the CRD fold, but usually lack the coordinated Ca^{2+}-ions and therefore generally lack the carbohydrate binding function. In analogy, this common fold is referred to as the C-

type lectin-like domain (CTLD)[46,47] and these proteins are thus named C-type lectin-like molecules. Although this rule applies to most proteins within the CTLD-family, some C-type lectin-like molecules display Ca^{2+}-independent carbohydrate recognition, such as Dectin-1[48], and other classical C-type lectins, which possess the structurally classical CRD fold, like DCIR, have so far not been shown to recognize any glycans structures[49].

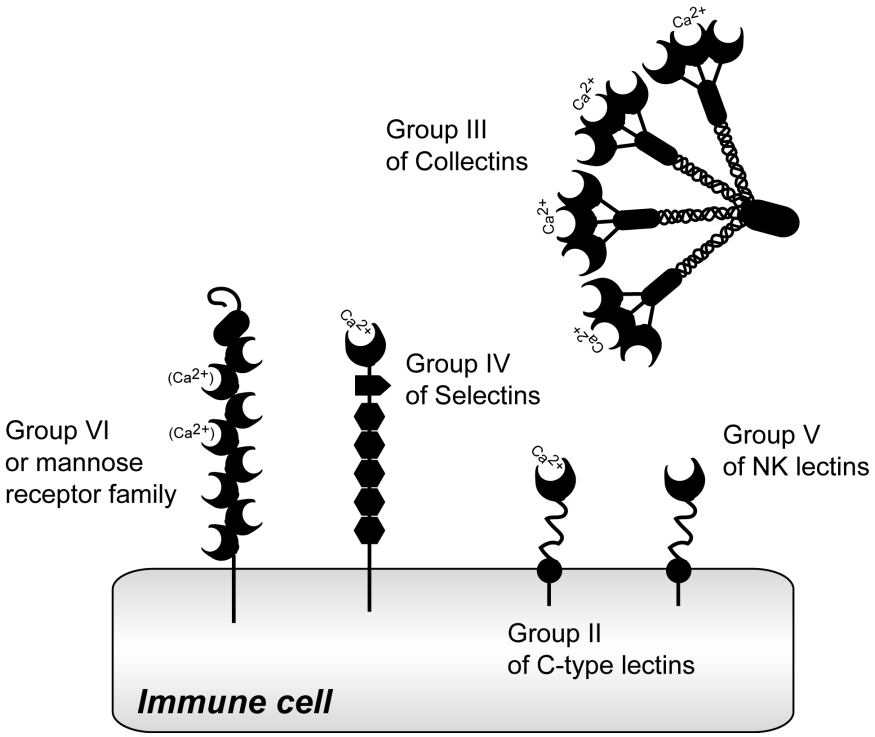

Figure 3. CTLD protein families in the immune system. Group II of C-type lectins encompasses the classical Ca^{2+}-dependent C-type lectins, such as DC-SIGN and MGL. Group III or the collectins are soluble Ca^{2+}-dependent PPRs in serum. Group IV or the selectins facilitate Ca^{2+}-dependent binding to sialylated Lewis structures, thereby mediating leukocyte migration. Group V of NK lectins are all located in the NK locus on human chromosome 12p13. These receptors display Ca^{2+}-independent binding to proteins or carbohydrates (Dectin-1). Group VI or the mannose receptor family includes four members with a similar tertiairy protein structure. Of group VI only MR and Endo180 interact with carbohydrates in a Ca^{2+}-dependent manner.

Classical C-type lectins can be divided into two categories based upon an amino acid motif involved in sugar recognition and coordination of the Ca^{2+}-ion. In mannose-type C-type lectins this is facilitated by the amino acid sequence Glu-Pro-Asn (EPN) within the CRD and in galactose-type lectins by the sequence Gln-Pro-Asp (QPD)[50]. Based upon this sequence predictions can be made regarding the carbohydrate recognition profile of a certain C-type lectin. Mannose-type lectins (containing the EPN-sequence) possess a basic specificity for mannose and/or fucose terminated glycans, whereas galactose-type lectins (containing QPD-sequence) recognize galactose or GalNAc terminated glycan structures. The secondary binding site fine-tunes this specificity so that each C-type lectin has its own unique glycan specificity. Furthermore, C-type lectins form oligomers within the cell membrane.

Oligomerization not only strengthens binding to a certain structure, it also limits binding to ligands with a complementary carbohydrate density and spacing[44]. Thus, carbohydrate recognition is primarily determined by the amino acid sequence of the CRD fold. However, it is strongly influenced by the oligomerization of the receptor and the spacing of carbohydrates on the ligand. So, C-type lectins, which have similar basic specificities for mannose, can still interact with a very diverse set of ligands.

C-type lectin	Nr. of CTLD	Expression pattern*	Carbohydrate specificity†	Self-ligands	Pathogens
MR [53-55]	8	DCs, Mϕs, lymphatic endothelium, LSEC	Mannose, Fucose, GlcNAc (CRD4), Sulfated glycans (CR)	(Pro)collagen, serum hydrolases, lutrophin, β-glucoronidase, myeloperoxidase, L-selectin	*M. tuberculosis*, HIV, *Candida albicans*
DEC-205 [56,57]	10	mono, DCs, lymphocytes, thymic eptithelium	none	Apoptotic thymocytes	?
Endo180 [58,59]	8	Fibroblasts, endothelial cells, Mϕs	Mannose, Fucose, GlcNAc (CRD2)	MMP-13, uPAR, Collagen	?
PLA_2R[60]	8	Muscle cells, alveolar epithelium	none	Collagen, Phospholipase A_2	?

Table 1. The human mannose receptor family of C-type lectins (group VI). Abbreviations: CR, cysteine-rich domain, HIV, human immunodeficiency virus, LSEC, liver sinusoidal endothelial cells, MMP-13, matrix metalloprotease-13, uPAR, urokinase plasminogen activator receptor. * expression patterns in human tissue, † Carbohydrate specificity of the human receptors

Recently, an extended classification of over 1000 vertebrate CTLD proteins, including the classical C-type lectins, in a total of 17 groups was proposed, based upon domain architecture and phylogenetic analysis in different species[45]. An overview of CTLD proteins important in the immune system is given in figure 3. The C-type lectins and C-type lectin-like proteins that are highly expressed by human APCs belong mainly to groups II, V and VI. Strikingly, some of these groups are found in distinct clusters within the human genome. On human chromosome 12p13, adjacent to the cluster of natural killer (NK) cell-specific CTLD proteins (group V), a part of group II of C-type lectin molecules, including DCIR and BDCA-2, is located[51]. Other clusters, belonging to group II, are found on chromosome 19p13, the so-called DC-SIGN cluster, and on chromosome 17p13 a cluster of galactose/GalNAc-specific lectins, such as asialoglycoprotein receptor (ASGP-R) and MGL, is found[45]. Based upon their chromosomal localization within the NK cell cluster CLEC-1, CLEC-2, DCAL-1,

DCAL-2 and Dectin-1 belong to group V of CTLD proteins[52].

Group VI or the mannose receptor family

Group VI or the mannose receptor family consists of four C-type lectin and C-type lectin-like proteins, MR, DEC-205, Endo180 and PLA_2R, that all contain a short cytoplasmic tail, a transmembrane region, 8 or 10 CTLD domains, a fibronectin type II (FNII) domain and an N-terminal cysteine-rich domain (Table 1)[61]. Of the four members, only MR and Endo180 show Ca^{2+}-dependent binding to mannose, fucose and GlcNAc residues via CRD 4 and 2 respectively[62,63]. A special feature of the MR is the Ca^{2+}-independent recognition of sulfated glycans via its cysteine-rich domain[64,65]. All members, with the exception of DEC-205, display carbohydrate-independent recognition of collagen via their FNII domains[66-68].

Groups II and V of C-type lectin and C-type lectin-like molecules

In contrast to the MR family that have type I transmembrane configuration, groups II and V of C-type lectin and lectin-like molecules are all type II transmembrane proteins with a similar tertiary structure consisting of a short cytoplasmic tail, a transmembrane region, a stalk region necessary for oligomerization and one CRD or CTLD domain. Group II, containing all the classical C-type lectins such as DC-SIGN and MGL, is large and heterogeneous; they undergo divergent evolution in different species, resulting in different homologues with distinct expression patterns and carbohydrate recognition profiles[69,70]. In contrast, group V of C-type lectin-like molecules is evolutionary young and therefore only found in higher vertebrates[45].

The CRDs of the classical C-type lectins have structurally similar folds, however subtle differences exist in their carbohydrate recognition profiles, probably due to modifications in secondary binding sites and/or oligomerization states of the individual lectins (Table 2). Although ASGP-R and MGL both belong to the galactose-type lectins, ASGP-R recognizes both GalNAc and galactose, while human MGL has an exclusive specificity for terminal GalNAc residues and very low or no affinity for galactose (Chapter 3)[71]. Also within the mannose-type C-type lectins this diversity is present. The homologues DC-SIGN and L-SIGN (77% identity at amino acid level[72]) have overlapping specificities, yet they possess lectin-specific ligands as well[73,74]. DC- SIGN and the MR both recognize high mannose-type glycans, however MR interacts with the terminal mannoses and DC-SIGN with more internal residues[113,114]. DCIR is the only human lectin that carries a related EPS sequence within its CRD, however its carbohydrate specificity has not been elucidated yet[49]. Strikingly, Dectin-1 recognizes β-glucans in a cation-independent manner and mutagenesis studies indicate that compared to the classical C-type lectins, distinct amino acids in the Dectin-1 CRD facilitate β-glucan binding[115].

C-type lectin function

In the last 5 years much progress has been made in the field of C-type lectin function within the immune system. Remarkably, several different C-type lectins share

common characteristics, including cell-cell and cell-matrix interactions, pattern recognition and internalization of antigens for presentation onto MHC class I and II. Furthermore, some C-type lectins possess signaling properties and are capable of modulating immune responses.
Many C-type lectins possess self-ligands, which can be glycosylated soluble or cellular molecules, or components of the extracellular matrix (Tables 1 and 2). MR

C-type lectin	Expression pattern*	Carbohydrate specificity†	Self-ligands	Pathogens
DC-SIGN[26,75-81]	DCs, specific Mϕ subsets	High mannose, Lewis antigens, GlcNAc (on LPS)	ICAM-2, ICAM-3, CD66a, MAC-1	HIV, HCV, Filoviruses, SARS, *M. tuberculosis, S. mansoni*
L-SIGN[73,74,80,82-85]	LSEC, lymph endothelium	High mannose, Lewis antigens (not Lewis X)	ICAM-2, ICAM-3	HCV, west nile virus, filoviruses, HIV, SARS
LSECtin[86,87]	LSEC, lymph endothelium	Mannose, fucose, GlcNAc	?	Filoviruses, SARS
ASGP-R[88-90]	hepatocytes	Terminal Gal/GalNAc	Serum glycoproteins	HCV, Filoviruses
MGL[71,91,92]	DCs, Mϕs	Terminal GalNAc	CD45	Filoviruses, *S.mansoni, C.jejuni*
Langerin[93-97]	LCs	High mannose, fucose, GlcNAc	Type I procollagen	HIV, *M. leprae*
Dectin-1[98-103]	LCs, DCs, pDCs, Mϕs, B cells, granulocytes, T cell subset	β1-3 and β1-6-linked glucans	T cell ligand	Fungi/yeast
Dectin-2[104,105]	Monocytes, LCs, pDCs	High mannose	?	?
DCIR[49]	DCs, Mϕs, B-cells, monocytes, granulocytes	?	?	?
BDCA-2[106]	pDCs	?	?	?
CLEC-1[52,107]	DCs, endothelium	none	?	?
CLEC-2[107-109]	Platelets, LSEC	none	?	Snake venom rhodocytin, HIV
DCAL-1[110]	DCs, B cells	none	$CD45RA^+$ T cells	?
DCAL-2[111] (MICL)	DCs, Mϕs, monocytes, granulocytes	none	?	?
SRCL[112]	Endothelium	Lewis X, Lewis A	?	?

Table 2. Overview of human group II and V of C-type lectin and C-type lectin-like molecules. LSEC, liver sinusoidal endothelial cells, HCV, hepatitis C virus, HIV, human immunodeficiency virus, SARS, severe acute respiratory syndrome coronavirus, * expression patterns in human tissue, † Carbohydrate specificity of the human receptors.

and ASGP-R participate in the homeostatic clearance of glycosylated proteins from circulation or body fluids[53,90]. DC-SIGN mediates cell-cell adhesion between DCs and a number of cell types, including ICAM-3 on naive T cells, allowing T cells to quickly scan the peptide repertoire presented in MHC molecules[78]. The DC-SIGN-ICAM-2 interaction facilitates DC migration and homing of DC precursors to the peripheral tissues[75]. Furthermore, engagement of MAC-1 and CD66a on neutrophils by DC-SIGN induces DC maturation, thereby establishing a molecular bridge between the innate and adaptive immune system[26,116].

Binding of soluble ligands, particles or pathogens to C-type lectins generally results in internalization of the antigens[97,117-120]. The internalized lectins travel through the endocytic pathway, releasing the cargo at low pH in either the endosomal or lysosomal compartments. Some C-type lectins, such as the MR, DEC-205 and ASGP-R, recycle back to the cell surface, whereas other C-type lectins are degraded together with their cargo[121]. Although targeting protein antigens to the C-type lectins may result in presentation of antigenic peptides in MHC class I and/or class II, this presentation does not always result in activation of adaptive immunity[117,122-126]. *In vivo* uptake of antigens via the murine DEC-205 receptor enhances antigen presentation in MHC class I 400 fold, however in the absence of a danger signal, responder $CD8^+$ T cells are deleted from the repertoire[42,119,121,123,125].

C-type lectins and especially the mannose-type lectins DC-SIGN and MR are targeted by a wide variety of microbes, including several viruses, bacteria, parasites and fungi (a selection of pathogens is listed in tables 1 and 2). Virus binding to C-type lectins can facilitate direct infection of the APC or sequester virus particles on the APC surface for subsequent delivery to target cells[76,127]. This phenomenon of viral transmission *in trans* was first reported for DC-SIGN and the human immunodeficiency virus (HIV)[128,129]. Moreover, infection of $CD4^+$ T cells is strongly enhanced when virus particles are captured and transmitted by DC-SIGN *in trans*.

The absence of immune activation after antigen binding to C-type lectins, led to the hypothesis that their primary function is to internalize antigen in the steady state for homeostasis control and that pathogens specifically target C-type lectins to evade specific immune responses in favor of pathogen survival[130]. In addition, C-type lectins are capable of modulating and fine-tuning immune responses. Combined triggering of Dectin-1 and TLR2 by fungal pathogens leads to the synergistic production of TNFα, IL-12 and reactive oxygen species (ROS)[14,48]. In contrast, collaborative recognition of *Mycobacterium tuberculosis* by DC-SIGN and TLR4 results in enhanced IL-10 production and a block in DC maturation[77]. DC-SIGN binding to Lewis-antigen positive *Helicobacter pylori* bacteria does not alter DC maturation; instead immune responses are skewed towards a Th2 phenotype[131]. Strikingly, triggering of DC-SIGN with a lipopolysaccharide mutant of *Neisseria meningitidis* results in Th1-mediated immunity (Chapter 10)[132]. Thus, the balance between TLR and C-type lectin activation on DCs may be instrumental in inducing either tolerance or immune activation.

MACROPHAGE GALACTOSE-TYPE LECTIN (CD301)

MGL homologues

So far, MGL orthologues have been found in rat, mice and man, whereas it is absent in fish (Table 3)[133]. MGL is most closely related to ASGP-R, the C-type lectin specific for hepatocytes[134]. The ASGP-R gene is located next to the MGL gene, suggesting that they might be derived from a common ancestor. ASGP-R forms heterodimers composed of two different protein subunits, whereas MGL is exclusively expressed as a homooligomer. All MGL orthologues contain within their carbohydrate recognition domain the QPD sequence predictive for galactose and/or GalNAc specificity. MGL is up to 60% identical to ASGP-R depending on the species and ASGP-R subunit, although identities are even higher when only the CRDs are compared. In the late 1980's Kawasaki *et al* discovered that rat peritoneal macrophages expressed a Ca^{2+}-dependent lectin, distinct from the hepatic ASGP-R, with specificity for galactose and GalNAc[135,136]. This C-type lectin mediated binding and uptake of cells and glycoproteins exposing terminal galactose residues[137]. Based on its binding properties the lectin was isolated and characterized as the rat MGL homologue, macrophage asialoglycoprotein-binding protein (M-ASGP-BP) or rMGL, a homooligomer (probably a hexamer or octamer) of 42 kDa subunits[138,139].

Mice contain two functional copies of the MGL gene, whereas in rats and humans only one copy of the gene is found[69,91]. The murine *Mgl* genes are located on chromosome 11, also tightly clustered to mouse ASGP-R. Although the *mMgl* promoter lacks a classical TATA box, it contains several binding sites for transcription factors that are generally found in cells of the monocyte/macrophage lineage, such as C/EBP, c-ETS, PU.1, Lyf-2 and AP-1, explaining the preferential expression of MGL in DCs and macrophages[140]. Although the mouse MGL homologues are highly similar in structure, their carbohydrate recognition profiles are quite distinct (see below). mMGL1 and mMGL2 are 79% identical on the nucleotide level and 91.5% identical in amino acid sequence. The major differences are found in the CRD and in the cytoplasmic regions, where mMGL2 contains an insertion of 14 amino acids. Their high homology and close genomic localization indicate that the original gene was duplicated only recently in mouse evolution. The human *mgl* gene was cloned from IL-2 treated monocytes and is 60% and 63% homologous to mMGL1 and rMGL respectively[141]. The human MGL (hMGL) mRNA is subject to alternative splicing, given rise to several variants resulting from deletions at three potential sites, however only one variant is expressed at high abundancy in APCs. A single nucleotide polymorphism has been found in exon 3 at a site that corresponds to amino acid 35 in the cytoplasmic tail and encodes for a Cys (CGC) or Arg (TGC) residue[91]. It is currently unknown whether the alternative splicing and the polymorphisms have any functional consequences for hMGL glycan specificity and ligand recognition. These topics would be interesting to pursue in future studies.

MGL expression pattern

rMGL was first isolated from thioglycolate-elicited peritoneal macrophages. In contrast resting peritoneal macrophages and macrophage-enriched splenocytes did not express significant rMGL-levels[135]. rMGL was strongly upregulated in bonemarrow-derived macrophages cultured with ConA-activated lymphocytes, suggesting that certain inflammatory conditions or macrophage subsets are associated with rMGL expression[142]. In accordance to these findings, rMGL expression was specifically upregulated in local macrophages in cardiac allografts in a chronic transplantation rejection model in rats[142,143]. In addition, rat lymph node DCs and rat interdigitating DCs possess receptors for galactose/GalNac[144]. This receptor probably represents rMGL, as MGL is expressed in both DCs and macrophages of mice and man.

Using the mMGL1/2-specific antibody ER-MP23[145] an expression pattern of mMGL was described on murine connective tissue APCs in close vicinity to epithelial surfaces. In addition, mMGL can be detected on macrophages and DCs in the subcapsular and interfollicular sinuses and in the T cell areas of mouse lymph nodes[146]. mMGL is expressed in several tissues including the skin, spleen, lung, large intestine, pancreas and thymus. In the lung mMGL is expressed in cells in connective tissue surrounding blood vessels and respiratory epithelia[147,148]. In mouse skin 60% of all leukocytes express CD11b, mMGL and the pan-macrophage marker F4/80. These cells are all localized to the dermis; resident mouse langerhans cells do not express mMGL[149]. Likely, a locally produced factor, which is not IL-4, IL-13 or an extracellular matrix component, triggers mMGL expression in these dermal phagocytes[145]. RT-PCR analysis on 13 different mouse tissues revealed a similar expression profile of mMGL1 and mMGL2. Antibody staining of mMGL on thioglycolate-induced peritoneal macrophages confirmed co-expressing of mMGL1 and mMGL2 on a single cell level. In addition, no differential expression of mMGL2 could be observed in mMGL1$^{-/-}$ mice compared to wild type mice[69].

Recently Raes *et al* identified mMGL1 and mMGL2 as markers for alternatively activated macrophages by employing a subtractive cDNA library from murine alternatively activated and classically activated macrophages[16]. In mouse models of allergic asthma and parasitic infections associated with alternative activation, mMGL expression was elevated on alveolar and peritoneal macrophages respectively. However, both hMGL and mMGL upregulation can occur independently of IL-4 signaling (Chapter 2)[92,145]. Also in autoimmune diabetes mMGL is implicated in disease progression via the recruitement of autoreactive lymphocytes to the pancreas[150]. Although mMGL^{+} macrophages are present in the pancreas of normal mice, their numbers are increased in the neonatal pancreas of non-obese diabetic (NOD) mice[151,152]. These mMGL^{+} histiocytic macrophages are characteristic of the initial phase of autoimmune diabetes before T cell infiltrates are detectable and they represent one of the first hematopoeitic cell populations to migrate towards and accumulate around the pancreatic islets[146].

hMGL is expressed by monocyte-derived DCs, whereas monocytes, lymphocytes,

plasmacytoid DCs or resident Langerhans cells do not express MGL[91,92,153,154]. RT-PCR analysis also revealed MGL mRNA expression in human granulocytes. Furthermore, hMGL is expressed at an intermediate stage of macrophage differentiation[155]. In human skin hMGL is expressed on dermal CD1a$^+$ APCs that probably represent a unique reservoir for the generation of both macrophages and immature DCs depending on local environmental conditions (Chapter 4[92] and [156]). Using newly generated hMGL-specific antibodies we could identify hMGL$^+$ APCs in small intestine, thymus and LN (Chapters 4[157] and 5). hMGL is not expressed in the human liver. Moreover, hMGL is upregulated during chronic inflammatory conditions such as rheumatoid arthritis on APCs located in the affected tissue (Chapter 4)[157].

Species	Homologue (alternative names)	Chromosomal localization	Expression pattern	Specificity
Human	hMGL (HML, DC-ASGPR)	17p13.1	Macrophages, immature DCs	Terminal GalNAc moieties, (Tn antigen, LacdiNAc)
Mouse	mMGL1	11 B3	Macrophages, immature DCs	Lewis X galactose, GalNAc
	mMGL2	11 B3	Macrophages, immature DCs	α- & β-GalNAc
Rat	rMGL (M-ASGP-BP, MGR, RML)	10q24	Macrophages, DCs?	Bi-antennary glycans with terminal galactose or GalNAc, Lewis A and Lewis X

Table 3. MGL orthologues in mouse, man and rat.

Carbohydrate specificities

In solid phase competition assays, binding of recombinant rMGL to galactose-BSA could be blocked by GalNAc and galactose with equal affinity[158]. In line with this, high affinity binding of asialoorosomucoid to rMGL-transfectants could be inhibited by both galactose or GalNAc-multimers, although galactose was a better competitor for asialoorosomucoid binding than GalNAc[159]. In a separate study using synthetic bi- and triantennary galactose ligands the highest affinity was observed for the trianntennary structure, probably related to the oligomerization of rMGL on the cell surface[160]. Recently Coombs *et al* probed the glycan array, developed by the Consortium for Functional Glycomics (http://www.functionalglycomics.org), with trimeric recombinant rMGL. rMGL bound a restricted set of glycans, displaying the highest affinity for bi-anntenary galactose and GalNAc-terminated structures. In addition, Lewis A, Lewis X and LDN glycans are recognized by rMGL[161].

Originally the carbohydrate recognition profile of recombinant mMGL1 was determined by comparing elution profiles of defined carbohydrate structures on immobilized lectin columns. Glycopeptides containing multiple terminal galactose or GalNAc structures had the highest affinity for mMGL1 as measured by relative

retardation times[162]. In solid phase competition assays mMGL1 binding to galactosylated poly-L-lysine could be blocked by carageenans, a group of sulfated polygalactans[163]. mMGL1 recognition was limited to carageenans as other sulfated glycoaminoglycans could not inhibit its function. Surprisingly, later studies reported quite distinct carbohydrate recognition profiles of recombinant mMGL1 and mMGL2 compared to the original identified profiles. Recombinant mMGL1 specifically recognized Lewis X structures, whereas mMGL2 had the highest affinity for α- and β-GalNAc and did not interact with Lewis X. In contrast to previous reports, no specific binding to other galactose-containing structures could be observed[69]. The observed differences in the specificities of mMGL were not confirmed using specific C-type lectin-transfectants or cells that naturally express the receptors, so currently the detailed carbohydrate recognition profiles of the mMGL proteins are still unknown.

Earlier studies using purified hMGL from COS-1 transfectants suggested a specificity for the monosaccharides galactose, GalNAc and even fucose[141]. In contrast, subsequent binding studies with recombinant MGL produced in a bacterial expression system identified a restricted GalNAc specificity, since recombinant hMGL bound with high affinity to peptides containing multiple Tn antigens, α-GalNAc residues linked to Ser or Thr[164]. Affinity increased with increasing numbers of Tn antigens, coinciding with the formation of recombinant hMGL trimers[164]. However, in these articles the hMGL specificity was not confirmed for cells that naturally express the receptor. We recently analyzed the carbohydrate recognition profile of the hMGL using glycan microarray profiling and hMGL-transfectants or hMGL$^+$ DCs, and identified an exclusive specificity for terminal α- and β-linked GalNAc residues that naturally occur as parts of glycoproteins or glycosphingolipids. Specific glycan structures containing terminal GalNAc moieties, expressed by the human helminth parasite *Schistosoma mansoni* as well as tumor antigens and a subset of gangliosides, were identified as ligands for MGL (Chapter 3)[71]. The carbohydrate specificities of the different MGL orthologues are summarized in table 3.

MGL in cellular interactions

Based on their high phagocytic capacity, mMGL$^+$ cells were originally classified as dermal macrophages[147,149]. However, mMGL$^+$ cells are able to migrate to the LN in a model of contact hypersensitivity after application of FITC-containing irritants, whereby the accumulation of mMGL$^+$ cells in the LN seems to positively correlate with the efficacy of sensitization[165,166]. Especially the more homogeneous mMGL2$^+$ subset endocytosed high quantities of FITC and displayed a clear DC morphology within the T cell cortex, hinting to a role for these cells in antigen presentation. Based on flowcytometric analysis, the FITC^{low} mMGL$^+$ APCs, located in the medullary and subcapsular sinus, are more heterogeneous and compass both DC subtypes and macrophages. Therefore, mMGL$^+$ dermal APCs most likely represent a heterogeneous population of DCs and macrophages.

The migration of mMGL$^+$ cells could be blocked by antibodies to either mMGL or IL-

1β[165], suggesting the involvement of mMGL in migration of APCs from the skin to the LN. Furthermore, intradermal injection of IL-1β resulted in a transient increase of mMGL$^+$ cells in the T cell area of the draining LN[167]. Strikingly, also migrating LCs transiently upregulate mMGL expression, however expression is rapidly lost upon LN arrival and only dermal-derived APCs retain their mMGL expression[145]. The expression pattern of mMGL in the murine LN displays a clear overlap with the distribution of mMGL-ligands, as probed with recombinant mMGL-protein[168]. The macrophage-specific receptor Sialoadhesin or Siglec-1 has been identified as a self-ligand for mMGL1. This counter-receptor for mMGL1 was isolated from lymph node lysates and possibly regulates the cellular localization of mMGL$^+$ cells within lymph nodes[169].

Recently, mMGL1 knockout mice were generated[170]. Similar to other C-type lectin knockout mice, mMGL1$^{-/-}$ mice do not display an overt phenotype[53,171]. Furthermore, expression of mMGL1 was not required for normal T and B cell development nor for allogeneic CD8$^+$ T cell responses. In irradiated embryos of mMGL1$^{-/-}$ mice a delayed removal of apoptotic cells was observed, hinting to a role for mMGL1 in the clearance of apoptotic cells[172]. Moreover, mMGL1$^{-/-}$ mice exhibited a reduced granulation formation in an air pouch model for tissue inflammation[173]. Granulation tissue is generated as a result of antigen-specific cellular immune responses associated with persistent infection or autoimmunity. Close to the site of antigenic stimulation an accumulation of mMGL1/2$^+$CD11c$^-$ macrophages was observed that secreted the proinflammatory cytokine IL-1α[174]. Injection of IL-1α in the air pouches of mMGL1$^{-/-}$ mice restored the formation of granulation tissue, suggesting that mMGL1$^+$ macrophages secrete this cytokine and thereby regulate the cellular influx and fibroblast activation necessary for the tissue remodeling[173]. If granulation formation was induced by an antigen-independent stimulus, no differences could be observed between wildtype and mMGL1 knockout mice[175].

Our work identified the first counter-receptors for hMGL on effector T cells and on lymphatic and sinusoidal endothelium. Via the hMGL-CD45 interaction, APCs can downregulate T cell activation, resulting in decreased cytokine and proliferative responses and even T cell death (Chapter 4)[157]. The ligand on endothelial cells has yet to be identified, although the specific binding suggests a role for hMGL in the homing to or retention of hMGL$^+$ APCs in lymphatic organs (Chapter 5).

MGL and tumor recognition

mMGL was originally isolated from tumoricidal macrophages[176,177], that are located in the lung metastases in a mouse model of ovarian cancer[148]. Strikingly, injection of an mMGL-transfected T cell line resulted in selective homing of these cells to metastatic tumor nodules, probably through the recognition of tumor-associated glycans[178]. Injection of blocking anti-mMGL antibodies significantly increased tumor loads at metastatic sites, confirming that mMGL$^+$ APCs contribute to the host defense against tumor metastasis[179].

hMGL might participate in the immune response to human adenocarcinomas as well

through the preferential recognition of the tumor-associated MUC1. hMGL is capable of distinguishing in patient material between MUC1 derived from healthy tissue and the tumor-associated MUC1 with an altered glycosylation pattern (Chapter 7). As hMGL is preferentially expressed by tolerogenic APCs (Chapter 2), binding of tumor MUC1 to hMGL likely promotes tumor progression instead of tumor rejection.

Pattern recognition by MGL

Compared to other mannose-type C-type lectins, MGL only interacts with a limited array of pathogens. Filoviruses cause hemorrhagic diseases with high mortality rates in humans and nonhuman primates. The *filoviridae* family can be divided into two genera, Marburg and the well known Ebola virus, that can be further subdivided into 4 distinct species, namely Zaire, Sudan, Ivory Coast and Reston. hMGL bound the highly O-glycosylated mucin-like domain within the envelope glycoprotein and promoted viral infectivity, by increasing viral attachment to natural cellular receptors[180]. The enhanced infectivity was positively correlated with the pathogenicity of the filovirus species used.

In addition, hMGL recognizes the helminth parasite *Schistosoma mansoni* and the causative agent of Guillain-barré syndrome, the bacterium *Campylobacter jejuni* (Chapters 3, 8 and 9)[71].

```
Human     MTRTYENFQYLENKVKVQGFKNGP----------LPLQSLLQRLCSGPCH
Rat       MTMAYENFQNLGSEEKNQEAGK----------APPQSFLCNILSWTH
Mouse 1     MIYENLQNSRIEEKTQEPGK----------APSQSFLWRILSWTH
Mouse 2   MTMRYENFQNLEREEKNQEMRNGDKKGGMESPKFALIPSQSFLWRILSWTH
```

Figure 4. Alignment of the cytoplasmic domains of the murine, rat and human MGL homologues[161]

MGL-mediated internalization and antigen presentation

MGL is able, like many other C-type lectins, to rapidly internalize after ligand binding[91,153,154]. Antibodies targeting hMGL are rapidly internalized from the cell surface[153]. All MGL homologues in mice, rat and man contain within their cytoplasmic tails the conserved YXXØ motif, where Ø denotes any amino acid with a bulky hydrophobic side chain. rMGL, mMGL2 and hMGL incorporate the YENF motif in the cytoplasmic region, whereas mMGL1 contains the highly homologous motif YENL[69]. Similar YXXØ motifs are found many different types of proteins, including the C-type lectins ASGP-R, DC-SIGN, Dectin-1, CLEC-1 and CLEC-2. The MR and DEC-205 possess other tyrosine-based motifs within their cytoplasmic domains, which likewise contribute to the internalization process[181]. In addition all MGL homologues contain a putative dileucine internalization motif within their cytoplasmic regions (Fig. 4). This motif is also present in the cytoplasmic tails of ASGP-R, L-SIGN and DC-SIGN[72,82].

In rMGL mutating or deleting the tyrosine, thereby disturbing the YXXØ motif, resulted in a ligand-induced internalization rate of about one-fourth of the wild-type

molecule, although wildtype and mutant rMGL were expressed at similar levels at the cell surface[182]. Similar to rMGL, the YENF is essential for internalization of hMGL. Disruption of the motif, by mutating the tyrosine into an alanine, completely abrogated hMGL-mediated endocytosis (Chapter 6). Even under steady state conditions a large proportion of the hMGL molecules resides intracellular in early endosomes and small vesicles that do not contain MHC II molecules, suggesting that hMGL might continuously recycle between the cell surface and the intracellular compartments. The mechanisms or pathways that MGL utilizes for internalization have not been elucidated, however antigens endocytosed by MGL are presented in MHC class II (Chapter 6).

MGL-mediated signaling/immunomodulation

So far no signaling properties have been established for MGL. However, the ASGP-R molecule, which contains some identical motifs, is phosphorylated on the tyrosine of the YXXØ motif and on serine residues, thereby regulating receptor distribution[183]. MGL could similarly be influenced by receptor phosphorylation. Furthermore, MGL phosphorylation might initiate intracellular signaling pathways.

DCs incubated with a glycosylation-defective mutant of *Campylobacter jejuni*, had higher expression of co-stimulatory molecules and secreted higher amounts of IL-6, TNFα and IL-10. As hMGL is at least one of the receptors on immature DCs for wildtype *C. jejuni*, these results could suggest that hMGL negatively regulates DC maturation (Chapter 8). Whereas it is still unclear whether hMGL-mediated signaling directly modulates APCs function, hMGL is involved in the negative regulation of effector T cells. Via the MGL-CD45 interaction tolerogenic APCs are able to suppress TCR-mediated T cell activation, resulting in reduced cytokine production and proliferation, providing another mechanism for APCs to control unwanted effector responses (Chapter 4)[157].

THESIS OUTLINE

At the initiation of this project little was known about detailed carbohydrate specificities or the immunological functions of many C-type lectins. Therefore, the scope of this research was to investigate how post-translational modifications of cellular ligands and pathogens regulate recognition by human C-type lectins and cellular communication of APCs with other cell types such as T cells and endothelial cells. Gradually, our focus shifted primarily to hMGL, the galactose-type lectin expressed on the APC surface, which converts the unique function of GalNAc recognition to the APCs.

Chapter 2 describes the generation of hMGL-specific monoclonal antibodies. Systematic analysis of hMGL expression patterns using these antibodies revealed that hMGL was upregulated on APCs with a tolerogenic phenotype, generated in the presence of the glucocorticoid dexamethasone[92]. To establish which immunological functions hMGL might fulfill, we probed the glycan microarray developed by the

Consortium for Functional Glycomics with recombinant MGL-Fc to elucidate the carbohydrate recognition profile of hMGL. In contrast to the mouse or the rat homologues, hMGL displays an exclusive specificity for terminal α- and β-GalNAc structures that naturally occur as post-translational modifications of glycoproteins or glycolipids (**Chapter 3**)[71]. Using this carbohydrate recognition profile predictions were made regarding counter-structures that might interact with MGL. This approach led to the identification of glycan structures containing terminal GalNAc moieties, expressed by the human helminth parasite *Schistosoma mansoni* as well as tumor antigens and a subset of gangliosides as ligands for hMGL. Next, we searched for self-ligands within the immune system and discovered that MGL specifically recognized with CD45 on effector T cells in a glycosylation-dependent manner, thereby negatively regulating TCR-mediated signaling (**Chapter 4**)[157]. Via the MGL-CD45 interaction, APCs revert excessive T cell activation by reducing cytokine responses, decreasing proliferation and the induction of T cell death. In addition, hMGL recognizes a counter-structure on sinusoidal and lymphatic endothelium within the lymph node and the thymus, hinting to a role for hMGL in the retention of APCs in these organs (**Chapter 5**). In **chapter 6** the hMGL-mediated internalization process was investigated. The YENF-motif in the hMGL cytoplasmic tail is essential for internalization and disruption of this motif, by mutating the tyrosine into an alanine, completely abrogated hMGL-mediated endocytosis. Furthermore, MGL can function as an antigen receptor for presentation on MHC class II molecules. The recognition of tumor antigens by hMGL was further explored in **chapter 7**. hMGL specifically discriminated between normal and tumor MUC1, recognizing only the tumor-associated form. The interaction of MGL-Fc with MUC1 present in tissue samples of primary colon carcinoma patients correlated strongly with expression of the Tn antigen, whereas no correlation was observed with other glycoforms of MUC1. In the chapters 8 to 10 we examined several C-type lectin-pathogen interactions and the functional consequences of such interactions on DC immunobiology. *Campylobacter jejuni*, the causative agent of Guillain-barré syndrome, is a unique bacterium capable of protein N-glycosylation. In **chapter 8** we demonstrate that hMGL can specifically recognize *C. jejuni* via the N-linked carbohydrate moieties composed of the bacterial sugar bacillosamine, one glucose and five GalNAc units. Loss of N-linked protein glycosylation in *C. jejuni* induced increased DC maturation and cytokine production as compared to the corresponding wildtype strain. DCs express a wide variety of C-type lectin receptors, which all can cooperate in pathogen recognition and subsequent internalization. *S. mansoni* derived soluble egg antigens (SEA) were bound by three C-type lectins on the DC surface, namely MR, DC-SIGN and hMGL. Binding of SEA to these C-type lectins results in targeting to MHC class II^+ vesicles, hinting to possible presentation of SEA, and skewing of naive T cell responses towards a Th2 phenotype (**Chapter 9**). Strikingly, binding of pathogens to DC-SIGN can also drive Th1-mediated responses. **Chapter 10** outlines the specific skewing of Th1-mediated immunity by the strong interaction of DC-SIGN with an *lgtB* lipopolysaccharide mutant of *Neisseria meningitidis*, that

contains a truncated outer core saccharide with a terminal GlcNAc residue[132]. Wildtype *N. meningitidis*, which is not bound by DC-SIGN, directed a mixed Th1/Th2 phenotype. Finally our results are integrated in a general discussion, presented in **chapter 11**.

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CHAPTER 2

DIFFERENTIAL REGULATION OF C-TYPE LECTIN EXPRESSION ON TOLEROGENIC DENDRITIC CELL SUBSETS

Sandra J. van Vliet, Ellis van Liempt, Teunis B.H. Geijtenbeek and Yvette van Kooyk

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ABSTRACT

Antigen presenting cells express high levels of the C-type lectins, which play a major role in cellular interactions as well as pathogen recognition and antigen presentation. The C-type lectin macrophage galactose-type lectin (MGL), expressed by dendritic cells and macrophages, mediates binding to glycoproteins and -lipids that contain terminal GalNAc moieties. To investigate MGL expression patterns in more detail we generated two new monoclonal antibodies and set up a quantitative real-time PCR analysis to determine MGL mRNA levels. MGL is not expressed by blood-resident plasmacytoid dendritic cells and thus represents an exclusive marker for myeloid-type antigen presenting cells. Dexamethasone treatment upregulated MGL expression on dendritic cells both at the protein and mRNA level in a time- and dose-dependent manner. In contrast, dendritic cells generated in the presence of IL-10 did not display enhanced MGL levels. Furthermore, dexamethasone and IL-10 also differentially regulated expression of other C-type lectins, such as DC-SIGN and mannose receptor. Our results demonstrate that, depending on the local microenvironment dendritic cells can adopt different C-type lectin profiles, which could have major influences on cell-cell interactions, antigen uptake and presentation.

INTRODUCTION

Professional antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages (Mϕs), are seeded throughout all peripheral tissues where they scan their surroundings for incoming pathogens or local environmental changes. Mϕs mainly represent traditional tissue-resident scavenging cells important in the effector phase of the immune response. Similar to Mϕs, DCs also play an essential role in uptake of self- or pathogenic antigens. DCs, once activated by proinflammatory stimuli or infectious pathogens, migrate towards the draining lymph node, where they initiate adaptive immunity[1,2]. Recently Pozzi *et al* demonstrated that also Mϕs can migrate to draining lymph nodes and activate naive $CD8^+$ T cells, although with lower efficiencies than DCs[3]. Next to immunity, DCs contribute to tolerance via the induction of T cell unresponsiveness or apoptosis or via the induction of regulatory T cells. These processes can be mimicked *in vitro* by the addition of glucocorticoids (GCs), such as dexamethasone, or by adding IL-10 to DC cultures[4,5].

Different DC lineages can develop from separate precursors or represent various activation states of a single subtype. DCs clearly possess a unique plasticity to adapt to environmental stimuli, leading to different functional phenotypes based on cell surface markers and production of cytokines and/or reactive metabolites[6]. Furthermore, recent evidence indicates that mouse splenic DCs and Mϕs renew from a common bone marrow progenitor that is able to develop into both subtypes depending on differential cytokine signaling[7]. In humans several pathways exist for the development of the different DC subtypes, such as Langerhans cells, plamacytoid

DCs and interstitial DCs, each requiring their own set of growth factors and/or cytokines[8].

As only a few surface proteins are expressed exclusively by DCs, new potential markers are required that can distinguish between the different phenotypic DC subtypes. One family of proteins, known to be differentially expressed by the various DC subsets, are the C-type lectins[9]. C-type lectins recognize specific carbohydrate moieties in a Ca^{2+}-dependent manner. They function as cell-cell adhesion molecules[10] and as pattern recognition receptors for pathogens[11]. Moreover, C-type lectins can internalize ligands, such as pathogens, but also self-glycoproteins for processing and presentation to T cells[12]. The C-type lectin macrophage galactose-type lectin (MGL) is expressed on *in vitro* cultured monocyte-derived DCs and Mϕs[13]. The carbohydrate recognition domain of MGL facilitates binding of terminal GalNAc-residues on glycoproteins, glyolipids or pathogens, in contrast to the well known mannose/fucose-specific lectins DC-SIGN and mannose receptor (MR)[14]. Although MGL was originally described to be a specific marker for cells at an intermediate stage of differentiation from monocytes to Mϕs[15], other reports demonstrate MGL to be expressed by dendritic cells and alternatively activated macrophages[16,17].

To further analyze the expression pattern of MGL on human DC subtypes, two new monoclonal antibodies (mAbs) directed against the C-type lectin MGL were generated. Our findings extend the knowledge on MGL expression patterns both at mRNA and protein level on functionally different immature DC subsets. We demonstrate that MGL is exclusively expressed by myeloid DCs and not by blood-resident plasmacytoid DCs. Only dexamethasone treatment, and not IL-10, can enhance MGL expression on tolerogenic DCs on both the protein and mRNA level. Furthermore, we show that expression of the C-type lectins MGL, DC-SIGN and MR is differentially regulated, suggesting that depending on the cellular environment, DCs can adopt various phenotypes with variable C-type lectin expression profiles.

MATERIALS AND METHODS

Cells and reagents

The cell lines CHO and CHO-MGL were maintained in RPMI containing 10% fetal calf's serum (Invitrogen, Carlsbad, CA). Immature DCs were cultured for 3-7 days from monocytes obtained from buffy coats of healthy donors (Sanquin, Amsterdam, the Netherlands) in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml, both from Biosource, Camarillo, CA). Plasmacytoid DCs were isolated from buffy coats using the BDCA-4 cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To some DC cultures dexamethasone (at concentrations indicated in the figures, Sigma-Aldrich, St. Louis, MO), IL-10 (10 ng/ml, Biosource) or LPS (100 ng/ml, Sigma-Aldrich) was added. Monocytes were stimulated with IL-2 (200 U/ml, Biosource), IL-4 (500 U/ml), IL-10 (10 ng/ml), dexamethasone (10^{-6} M) or GM-CSF (1000 U/ml).

Generation of anti-MGL mAbs
Balb/c mice were immunized three times with recombinant MGL-Fc[14]. After the final boost, spleen cells were fused with SP2/0 cells at a 1:1 ratio using PEG. Hybridoma supernatants were screened for the presence of anti-MGL mAbs on CHO-MGL transfectants. After two rounds of cloning two hybridomas (1G6.6 and 18E4) were obtained that specifically recognize MGL.

Flow cytometry
Cells were incubated with primary antibody (5 μg/ml), followed by staining with a secondary FITC-labeled goat anti-mouse antibody (Zymed, San Francisco, CA) and analyzed on FACScalibur (BD Pharmingen, San Diego, CA). The following mAbs were used: isotype control 28-14-8 (mouse anti-mouse H2-D^b), AZN-D1 (DC-SIGN) and 3.29.B1 (mannose receptor)[12]. The anti-MGL antibody MLD-1 was kindly provided by dr. T. Irimura.

Immunohistochemistry
Cryosections of healthy humans tissues (7μm) were fixed with 100% aceton and stained with primary antibodies (10 μg/ml) for 1 hour at 37°C. Sections were counterstained with goat anti-mouse IgG2a-specific Alexa 488 antibodies (Molecular probes, Eugene, OR) and analyzed by fluorescence microscopy.

mRNA isolation and cDNA synthesis
mRNA was isolated by capturing poly(A+) RNA in streptavidin-coated tubes with an mRNA Capture kit (Roche, Basel, Switzerland) and cDNA was synthesized with the Reverse Transcription Sytem kit (Promega, Madison, WI) following manufacturer's guidelines. Cells (0.1 x 10^6/well) were washed twice with ice-cold PBS and harvested with 100 μl lysis buffer. Lysates were incubated with biotin-labeled oligo(dT)$_{20}$ for 5 min at 37°C. The mix was transferred to streptavidin-coated tubes and incubated for 5 min at 37°C. After washing 3 times with 200 μl washing buffer, 30 μl of the reverse transcription mix (5 mM $MgCl_2$, 1x reverse transcription buffer, 1 mM dNTP, 0.4 U recombinant RNasin ribonuclease inhibitor, 0.4 U AMV reverse transcriptase, 0.5 μg random hexamers in nuclease-free water) were added to the tubes and incubated for 10 min at room temperature followed by 45 min at 42°C. To inactivate AMV reverse transcriptase and separate mRNA from the streptavidin-biotin complex, samples were heated at 99°C for 5 min, transferred to microcentrifuge tubes and incubated on ice for 5 min, diluted 1:2 in nuclease-free water, and stored at -20°C.

Quantitative real-time PCR
Oligonucleotides have been designed by using computer software Primer Express 2.0 (Applied Biosystems, Foster City, CA). Primers were synthesized by Invitrogen (Invitrogen). Primer specificity was computer tested (BLAST, National center for Biotechnology Information) by homology search with the human genome and later confirmed by dissociation curve analysis. Primers used in this study; GAPDH: Fwd

5′-CCATGTTCGTCATGGGTGTG and Rev 5′-GGTGCTAAGCAGTTGGTGGTG; MGL: Fwd 5′-TACACCTGGATGGGCCTCAG, Rev 5′-TGTTCCATCCACCCA CTTCC, DC-SIGN: Fwd 5-AACAGCTGAGAGGCCTTGGA and Rev 5′-GGGACCATGGCCAAGACA. PCR reactions were performed with SYBR green method in an ABI 7900HT sequence detection system (Applied Biosystems). The reactions were set on a 96-well plate by mixing 4 μl of the 2 times concentrated SYBR Green Master Mix (Applied Biosystems) with 2 μl of the primer solution containing 5 nmol/μl of both primers and 2 μl of a cDNA solution. The cDNA synthesis product was diluted 1:2 in nuclease-free water. The thermal profile for all the reactions was 2 min at 50°C, followed by 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min 60°C. The fluorescence monitoring occurred at the end of each cycle. The Ct value is defined as the number of PCR cycles where the fluorescence signal exceeds the threshold value, which is fixed above 10 times the standard deviation of the fluorescence during the first 15 cycles and typically corresponds to 0.2 relative fluorescence units. This threshold is set constant throughout the study and corresponds to the log linear range of the amplification curve. The normalized amount of target, or relative abundance, reflects the relative amount of target transcripts with respect to the expression of the endogenous reference gene. The endogenous reference gene chosen was GAPDH.

RESULTS

To generate specific mAbs reactive with the C-type lectin MGL, Balb/c mice were immunized three times with purified MGL-Fc. After the final boost spleen cells were fused with the SP2/0 myeloma. Hybridoma supernatants were screened by FACS for positive staining on CHO-MGL transfectants and negative reactivity with the parental CHO cells. CHO-MGL transfectants expressed high levels of MGL, as determined by flowcytometric analysis with a previously described anti-human MGL mAb (Fig. 1A and [15]). After two rounds of cloning two MGL-reactive hybridomas, 1G6.6 and 18E4, of the IgG2a isotype were obtained (Fig. 1B). To further confirm the specificity of these hybridomas, human skin, lymph node and liver sections were stained with the new mAbs (Fig. 1C). In agreement with earlier reports[15], MGL^+ cells were readily detected in the dermis of human skin. No cross-reactivity with the most closely related human homologue of MGL, the liver-specific C-type lectin ASGP-R, was observed, confirming the exclusive specificity of the generated mAbs for MGL (Fig. 1C). In addition, human lymph node contained numerous MGL^+ cells within the outer zones of the paracortex. Similar results were obtained for both anti-MGL mAbs.

The availability of the anti-MGL mAbs allowed us to investigate MGL expression levels in various human DC subsets. Plasmacytoid DCs (pDCs) represent a distinct DC population that is characterized by the markers BDCA-2 and BDCA-4[18]. To investigate whether next to myeloid-type DCs, also pDCs express MGL, we isolated pDCs from human buffy coats. Although the isolated pDCs clearly express the pDC

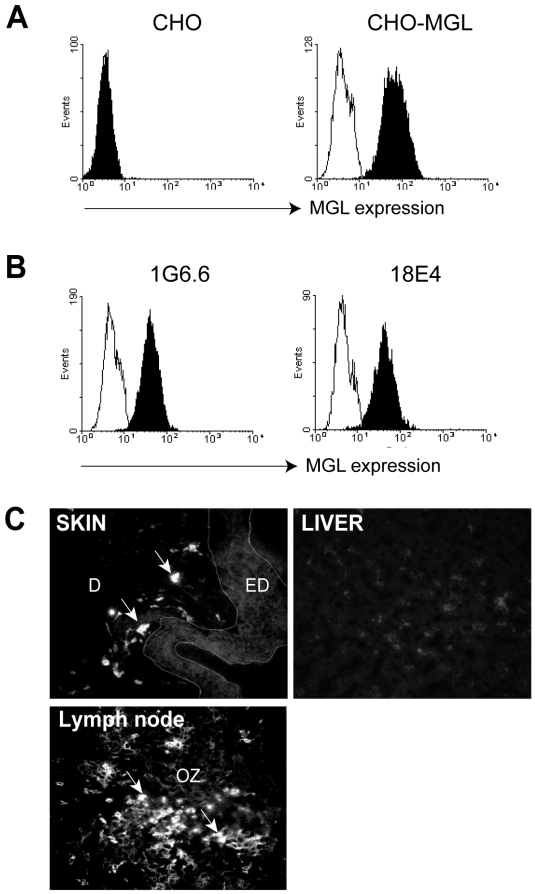

Figure 1. Generation of hybridomas producing MGL-specific mAbs. (A) Stable transfectants of CHO-MGL express high levels of MGL as assessed by flow cytometric analysis with a previously described anti-MGL antibody (kindly provided by dr. T. Irimura). Open histograms represent isotype control staining, filled histograms represent MGL staining. (B) After two round of cloning two MGL-specific hybridoma's (1G6.6 and 18E4) were obtained that specifically stain CHO-MGL transfectants. Open histograms represent isotype control staining, filled histograms represent MGL staining on CHO-MGL by the mAbs 1G6.6 and 18E4. (C) New MGL mAbs recognize MGL$^+$ cells in skin and lymph node, whereas no crossreactivity was observed with the highly homologous liver-specific molecule ASGP-R. Human tissue sections of skin, lymph node and liver were stained with 18E4 mAbs. ED, epidermis, D, dermis, OZ, outer zones of the paracortex. Arrows indicate MGL$^+$ cells. Original magnification 400x. One representative experiment out of three is shown.

marker BDCA-2, no MGL expression could be detected by either 18E4 or 1G6.6 mAbs (Fig. 2). No MGL mRNA could be detected in pDCs by quantative real-time PCR analysis (data not shown). These results demonstrate that MGL is specifically expressed by myeloid DCs and not by pDCs.

To determine the factors capable of inducing MGL expression, monocytes were treated with several cytokines and the glucocorticoid dexamethasone. At day 3 monocytes were analyzed by flow cytometry for the expression of MGL (Table 1). To control for the effectiveness of the cytokine treatment, DC-SIGN and MR were included in the analysis. DC-SIGN expression has been reported to be induced by IL-4[19], whereas MR expression is upregulated by IL-4, as well as GM-CSF and dexamethasone[20]. In contrast, MGL expression was not observed on the cell surface after IL-4 treatment, although MGL mRNA was elevated[17]. Mϕs generated by culturing monocytes in the presence of GM-CSF and IL-4/GM-CSF-cultured immature DCs expressed high levels of MGL. In contrast, LPS-matured DCs completely lacked MGL expression, both on the protein and on the mRNA level, whereas expression of DC-SIGN or the MR could still be observed (Table 1). Although the mouse homologue of MGL was originally cloned from IL-2 treated monocytes[13], we could not detect enhanced MGL expression on IL-2 treated human monocytes. Strikingly, a 3 day treatment with a single dose of the glucocorticoid dexamethasone could enhance MGL expression substantially (Table 1). In conclusion, the expression of the C-type lectins MGL, DC-SIGN and MR are

differentially regulated, suggesting that depending on the cellular environment, DCs can adopt various phenotypes with variable C-type lectin expression profiles.
DCs cultured in the presence of IL-10 or dexamethasone are generally considered to be tolerogenic DCs capable of inducing tolerance instead of immunity[5]. As MGL expression seems to be differentially regulated by IL-10 or dexamethasone, a single dose of these stimuli was added at the start of DC differentiation and the generated DCs were evaluated at day 4 for MGL, DC-SIGN and MR expression. Strikingly, IL-10 and dexamethasone cultured immature DCs display differential expression profiles of C-type lectin receptors (Fig. 3A and B). Whereas MGL expression is slightly decreased on immature DCs treated with IL-10, it is highly upregulated by dexamethasone exposure. In contrast to MGL, DC-SIGN and MR are downregulated by the addition of dexamethasone. DCs treated with IL-10 acquire higher levels of MR, where as the DC-SIGN expression remains unchanged. These results indicate that IL-10 tolerogenic DCs are characterized as MGL^{low}, $DC\text{-}SIGN^{high}$ and MR^{high}, whereas dexamethasone-cultured DCs are MGL^{high}, $DC\text{-}SIGN^{low}$ and MR^{high}.

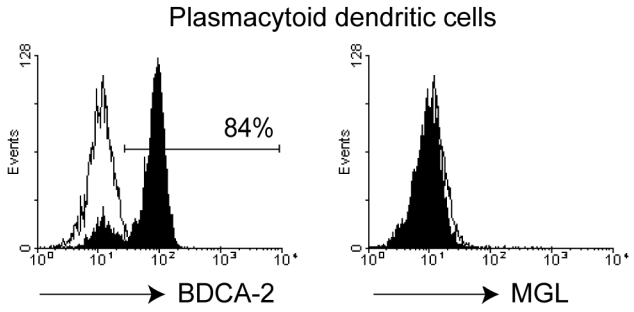

Figure 2. MGL is not expressed by plasmacytoid DCs. Highly enriched plasmacytoid DCs (84% pure) were stained for BDCA-2 and MGL by flow cytometry. Open histograms indicates isotype control staining, filled histograms represent BDCA-2 or MGL staining. One representative experiment is shown.

To investigate whether the enhanced MGL expression observed after dexamethasone treatment is due to enhanced cell surface expression from intracellular pools[21] or from enhanced MGL translation, immature DCs were cultured in the presence of the indicated concentrations of dexamethasone. At day 4 DCs were harvested and MGL and DC-SIGN mRNA levels were determined by quantitative real-time PCR. Compared to untreated DCs, MGL mRNA levels are clearly upregulated in dexamethasone-exposed DCs in a dose-dependent manner. Enhanced MGL mRNA levels were observed in immature dexamethasone-treated DCs at dexamethasone concentrations higher than 10^{-8} M (Fig. 3C). In contrast, DC-SIGN expression was downregulated at the mRNA level by dexamethasone exposure at concentrations higher than 10^{-8} M (Fig. 3D). These results confirm that MGL expression is enhanced at the transcriptional level and is not the result of enhanced cell surface expression from intracellular stores. To investigate how MGL expression is regulated in time after dexamethasone exposure, a single dose of dexamethasone (10^{-6} M) was added at

Stimuli	DC-SIGN[a]	MGL	Mannose receptor
IL-2	-	-	-
IL-4	+	+[b]	+
IL-10	-	-	-
GM-CSF	-	+	+
IL-4/GM-CSF	+	+	+
IL-4/GM-CSF/LPS	+	-	+
Dexamethasone	-	+	+

Table 1. C-type lectin expression on stimulated monocytes. [a]Monocytes were cultured for three days in the presence of the indicated cytokines, glucocorticoids or combinations, after which C-type lectin expression was determined by flow cytometry (DC-SIGN, MGL and MR) and real-time PCR (DC-SIGN and MGL). + indicates induction of C-type lectin expression compared to unstimulated monocytes, - no expression. [b]Upregulation only observed at the mRNA level.

the onset of DC differentiation and MGL expression was followed in time by real-time PCR analysis. At every timepoint measured, MGL mRNA levels were higher in the dexamethasone-exposed immature DCs compared to the untreated immature DCs (Fig. 3E). In the dexamethasone DCs MGL mRNA levels peaked at day 5 and slowly started to decline thereafter, confirming that a single dose of dexamethasone is sufficient to selectively upregulate MGL expression for at least one week. In summary, dexamethasone but not IL-10, enhances MGL expression at the mRNA levels in a time- and dose-dependent manner.

DISCUSSION

MGL is a C-type lectin that participates in the binding and uptake of glycoproteins and –lipids by immature DCs and Mϕs[22]. Recently, the carbohydrate recognition profile of MGL was elucidated, consisting of unique specificity for terminal GalNAc-residues[14]. As most other C-type lectins expressed on the cell surface of antigen presenting cells, such as DC-SIGN and MR, have mannose/fucose specificity[23,24], MGL confers the specialized function of GalNac recognition to the APCs. Therefore, a more detailed examination of MGL expression patterns and specific stimuli controlling MGL expression are warranted, to establish under which environmental conditions MGL is specifically expressed and which APCs may exhibit its function.
mAbs are unsurpassed tools to study not only receptor expression, but also allow the identification of receptor involvement in biological processes and subsequent pathways induced by receptor activation. Here the generation of two new mAbs directed against human MGL is described (Fig. 1). With the use of these new mAbs the expression pattern of MGL on different human DC subsets could be assessed. Human Langerhans cells and interstitial DCs are derived from a common precursor, whereas human pDCs develop along a separate pathway[8]. Although the expression of MGL on DCs and Mϕs has been well documented[14,15], the presence of MGL on human pDCs has not been investigated. Here we show that MGL is not expressed

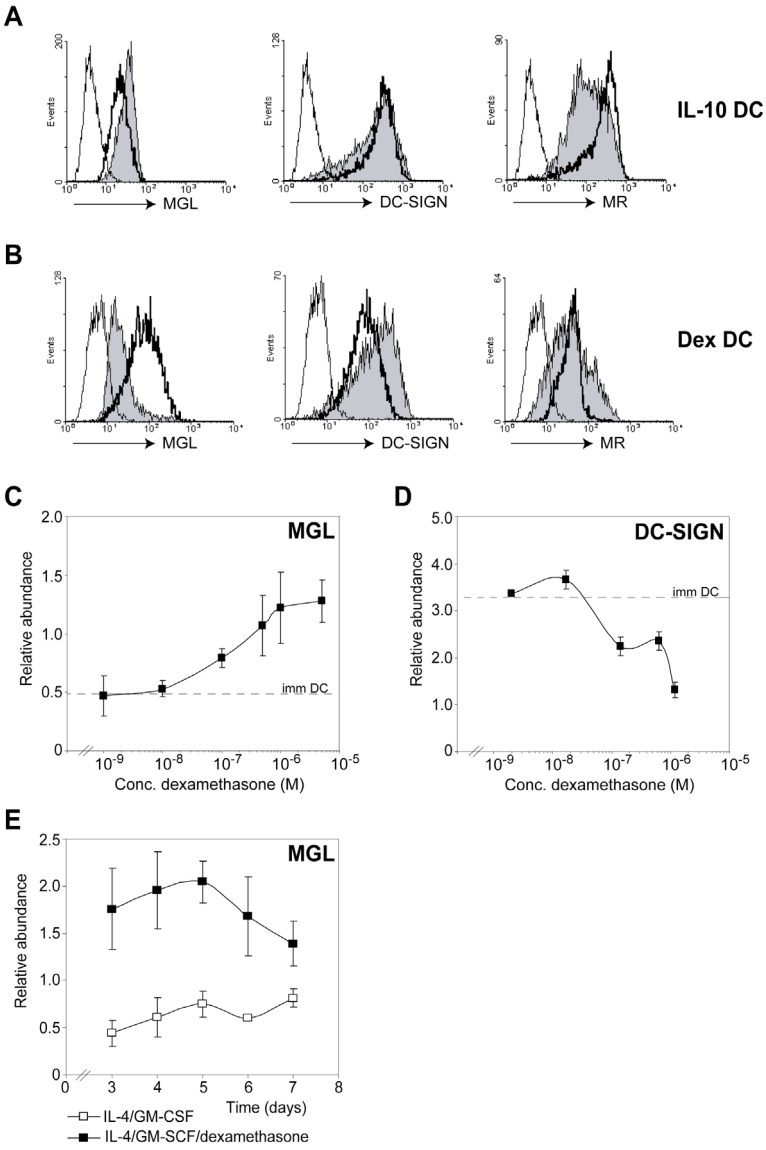

Figure 3. Dexamethasone treatment enhances MGL expression during DC differentiation. Immature DCs were generated from monocytes by culturing for 4 days with IL-4 and GM-CSF in the presence or absence of IL-10 (A) or dexamethasone (B-D). (A) Cell surface expression of MGL, DC-SIGN and MR was determined by FACS analysis on immature DCs (filled histograms) and IL-10 cultured DCs (10 ng/ml, solid line). The isotype control staining is indicated by the thin line. (B) Cell surface expression of MGL, DC-SIGN and MR was determined by FACS analysis on immature DCs (filled histograms) and dexamethasone cultured DCs (10^{-6} M, solid line). The isotype control staining is indicated by the thin line. (C) Dose response curve of dexamethasone-induced MGL expression. Monocytes were cultured with IL-4/GM-CSF and dexamethasone at the indicated concentrations. At day 4 cells were harvested and MGL mRNA levels were determined by quantitative real-time PCR. The dashed line indicates the MGL mRNA level in untreated immature DCs. (D) Dose response curve of dexamethasone-mediated inhibition of DC-SIGN expression. Monocytes were cultured with IL-4/GM-CSF and dexamethasone at the indicated concentrations. At day 4 cells were harvested and DC-SIGN mRNA levels were determined by quantitative real-time PCR. The dashed line indicates the DC-SIGN mRNA level in untreated immature DCs. (E) Kinetics of dexamethasone-induced MGL expression. Monocytes were cultured with IL-4/GM-CSF in the presence or absence of dexamethasone (10^{-6} M) for 3-7 days. At indicated timepoints cells were harvested and MGL mRNA levels were determined by quantitative real-time PCR. One representative experiment out of three is shown.

either on the protein or the mRNA level by human pDCs (Fig. 2). MGL thus represents an exclusive marker for myeloid-type antigen presenting cells, similar to DC-SIGN and the MR[25].

Factors capable of driving the acquisition of MGL on monocytes include GM-CSF and dexamethasone. IL-4 induces MGL expression only at the mRNA level (Table 1 and [17]). IL-4/GM-CSF generated immature DCs expressed high levels of MGL, DC-SIGN and MR. In contrast, LPS-matured DCs completely lacked MGL expression,

both on the protein as well as the mRNA level, whereas MR and DC-SIGN were still expressed albeit at slightly lower levels (Table 1 and [12]). Furthermore, immature DCs cultured in the presence of dexamethasone display enhanced levels of MGL. By contrast IL-10 was identified as a negative regulator of MGL expression on immature DCs (Fig. 3A and B). Both IL-10 and dexamethasone inhibit the immunostimulatory capacities of antigen-presenting DCs. IL-10 cultured DCs have an impaired capacity to stimulate alloreactive T cells, due to lower expression of costimulatory molecules and a decreased production of IL-12[26,27]. Similarly, dexamethasone treatment blocks DC maturation at an early stage, resulting in a more immature phenotype. Dexamethasone-DCs express lower levels of costimulatory molecules, whereas the expression of molecules involved in antigen uptake and cell adhesion is elevated[28]. Although the endocytic capacity of dexamethasone-DCs was increased, subsequent antigen presentation to T cells occurred with a much lower efficiency[29]. Therefore, IL-10- or dexamethasone-treated DCs are considered to be tolerogenic DCs[5].

GCs, like dexamethasone, are widely used as anti-inflammatory and immunosuppressive agents in the therapy of allergy or autoimmune diseases. *In vivo*, GCs belong to the family of steroid hormones whose production by thymic epithelial cells and the adrenal gland is increased during a stress response[30] and as a natural feedback loop during an immune response to avoid potential damage to the host[31]. GCs exert their many immunoregulatory effects via the GC receptor, which is widely expressed in cells of the immune system. GCs penetrate the cell and bind to the cytosolic GC receptor after which the complex translocates to nucleus for positive and negative gene regulation[32].

The reduced ability of dexamethasone-cultured DCs to stimulate T cells is contributed to the induction of the transcriptional regulator Glucocorticoid-Induced Leucine Zipper (GILZ)[33]. Inhibition of GILZ reverses the negative effects on cytokine production and costimulatory molecule expression. It seems unlikely that MGL expression is regulated via GILZ, as both IL-10 and dexamethasone are capable of inducing GILZ expression[33]. Moreover, GILZ functions as a potent inhibitor of the transcription factor AP-1[34]. Although the MGL promoter contains two AP-1 sites[35], MGL levels are enhanced by dexamethasone, suggesting that MGL expression is not driven by AP-1.

Strikingly, for all the C-type lectins investigated in this report, namely MGL, DC-SIGN and MR, the expression is differentially regulated on *in vitro* cultured DCs. These results indicate that also *in vivo* different APC subsets exist that exhibit unique C-type lectin expression patterns. C-type lectins participate in cell-cell communication, pattern recognition, as well as antigen uptake for presentation in MHC class I and II[10-12]. Changes in the local environment and thus on C-type lectin profiles could therefore greatly influence many aspects of the immune response that are mediated by C-type lectins. Future studies on the mechanisms controlling MGL expression are required to determine whether the presence of MGL correlates with distinct stages of an immune response or with certain pathological conditions.

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CHAPTER 3

CARBOHYDRATE PROFILING REVEALS A DISTINCTIVE ROLE FOR THE C-TYPE LECTIN MGL IN THE RECOGNITION OF HELMINTH PARASITES AND TUMOR ANTIGENS BY DENDRITIC CELLS

Sandra J. van Vliet, Ellis van Liempt, Eirikur Saeland, Corlien A. Aarnoudse, Ben Appelmelk, Tatsuro Irimura, Teunis B.H. Geijtenbeek, Ola Blixt, Richard Alvarez, Irma van Die and Yvette van Kooyk

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ABSTRACT

Dendritic cells are key to the maintenance of peripheral tolerance to self-antigens and the orchestration of an immune reaction to foreign antigens. C-type lectins, expressed by dendritic cells, recognize carbohydrate moieties on antigens that can be internalized for processing and presentation. Little is known about the exact glycan structures on self-antigens and pathogens that are specifically recognized by the different C-type lectins and how this interaction influences dendritic cell function. We have analyzed the carbohydrate specificity of the human C-type lectin MGL using glycan microarray profiling and identified an exclusive specificity for terminal α- and β-linked GalNAc residues that naturally occur as parts of glycoproteins or glycosphingolipids. Specific glycan structures containing terminal GalNAc moieties, expressed by the human helminth parasite *Schistosoma mansoni* as well as tumor antigens and a subset of gangliosides, were identified as ligands for MGL. Our results indicate an endogenous function for dendritic cell expressed MGL in the clearance and tolerance to self-gangliosides, and in the pattern recognition of tumor antigens and foreign glycoproteins derived from helminth parasites.

INTRODUCTION

Antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages (Mϕs) are the key players in the initiation and control of innate and adaptive immune responses. In order to perform their function both DCs and Mϕs are equipped with a full array of specialized receptors, including adhesion receptors, costimulatory molecules and several pattern recognition receptors, such as C-type lectins and Toll-like receptors[1].

Within the last few years several C-type lectins, which recognize specific carbohydrate structures in a Ca^{2+}-dependent manner, C-type lectin-like molecules and selectins have been identified on both DCs and Mϕs, including DC-SIGN[2], mannose receptor, langerin, DEC205 and L-selectin[3]. Specific glycosylation patterns regulate leukocyte homing and trafficking processes within the immune system[4]. Changes in glycosylation can similarly control the interaction of DCs with other cell types, thereby modulating migration and immune responses[5]. Most C-type lectins, with the exception of DC-SIGN and mannose receptor, have been poorly characterized with respect to their carbohydrate specificity and function within the immune system. It has been postulated that C-type lectins function in cell-cell adhesion, antigen recognition and serve as signaling molecules influencing the balance between tolerance and immunity[6]. C-type lectin stimulation can either enhance or inhibit TLR signaling thereby modulating DC phenotype and outcome of immune responses[7,8]. The cytoplasmic tail of C-type lectins often contains signaling motifs or internalization motifs for processing of antigens[9].

Predictions on carbohydrate specificities of C-type lectins for either galactose-type or mannose-type glycans can be made based on the primary amino acid sequence.

Knowledge on the exact carbohydrate recognition profile is essential to understand the importance of these receptors in immune related functions. Studies on carbohydrate recognition have long been hampered due to the complexity of glycan synthesis and the limited availability of isolated or synthesized glycans.
Recognition of mannose- and fucose structures by DC-SIGN and mannose receptor on DCs has been widely investigated. However, studies on galactose or GalNAc recognition by DCs are limited. One galactose-type C-type lectin has been reported to be expressed by human DCs, namely the macrophage galactose-type lectin (MGL, also called DC-ASGPR or HML)[10-12]. MGL is a member of the type II family of C-type lectins. MGL and is expressed on human and mouse immature DCs and Mϕs in skin and lymph node[13]. No natural ligand or function for MGL has been established yet[14]. Mice contain two functional copies of the MGL gene, mMGL1 and mMGL2, whereas in humans only one MGL gene is found. mMGL1 and mMGL2 have different carbohydrate specificities for respectively Lewis X and α/β-GalNAc structures[15]. Earlier studies on COS-1 transfectants of MGL suggested a specificity for the monosaccharides galactose and GalNAc[12]. In contrast, recombinant MGL produced in a bacterial expression system displayed restricted binding to GalNAc[16]. The recognition of more complex oligosaccharides by human MGL has not been thoroughly investigated yet.
To gain more insight in the function and carbohydrate specificity of MGL and galactose/GalNAc recognition by human DCs, we set out to identify the carbohydrate recognition profile of human MGL by using glycan microarray screening. The glycan array was developed by the Consortium for Functional Glycomics (http://www.functionalglycomics.org) and consists of more than one hundred synthetic and natural glycan structures. The use of glycan arrays for the elucidation of carbohydrate recognition profiles of individual C-type lectins has been applied to selectins, langerin and DC-SIGN homologues[17-19].
Using an MGL-Fc chimeric protein, we identified oligosaccharides containing terminal α- or β-linked GalNAc residues as high affinity ligands for MGL. Such terminal GalNAc residues can be part of protein N- or O-linked glycans, or glycosphingolipids. Identification of this specificity led to the discovery of glycan antigens within egg glycoproteins of the pathogenic helminth *Schistosoma mansoni* as counter-structures for MGL. In addition MGL strongly interacted with tumor cells in an α-GalNAc-specific manner. Our results strongly implicate a role for MGL in recognition of self-gangliosides, tumor antigens and pathogenic helminths by DCs.

MATERIALS AND METHODS

Cells

The adenocarcinoma cell lines SW948, SKBR3 and ZR75-1, CHO and CHO-MGL cells and the melanoma cell lines BLM, FM3.29, FM6, SK23mel, 90.07 and 00.09 were maintained in RPMI or DMEM medium (Invitrogen, Carlsbad, CA) containing 8-10% fetal calf's serum. Immature monocyte derived DCs were cultured for 5-7 days from

monocytes obtained from buffy coats of healthy donors (Sanquin, Amsterdam) in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml).

Antibodies and reagents

The following mAbs were used: MLD-1 (anti-MGL[10]), AZN-D1 (anti-DC-SIGN), SMLDN1.1 and SMFG4.1 (anti-LDN[20] and anti-LDNF, respectively, kindly provided by dr. A. Nyame and dr. R. Cummings, University of Oklahoma Health Sciences Center, USA) and 6H3 (anti-Lewis X). Biotinylated polyacrylamide coupled glycoconjugates were obtained from Lectinity (~20% substitution, Lappeenranta, Finland). Crude *Schistosoma mansoni* soluble egg antigen (SEA) extract was prepared as previously described (kindly provided by Dr. F. Lewis)[21]. Forssman glycolipid was a kind gift from Dr. R. Geyer (University of Giessen, Germany). DC-SIGN-Fc has been described previously[22]. The peroxidase labeled or biotinylated lectins Con A (*Canavalia ensiformis),* HPA (*Helix pomatia),* MAA (*Maackia amurensis*), PNA (*Arachis hypogaea*), SBA (*Glycin max*), SNA (*Sambucus nigra*) and WGA (*Triticum vulgaris*) were obtained from Sigma-Aldrich (St. Louis, MO).

Isolation and expression of the cDNA encoding MGL and MGL-Fc

The cDNA encoding human MGL[12] was amplified on total RNA from immature DCs, cloned into expression vector pRc/CMV and confirmed by sequence analysis. Stable CHO transfectants were generated using lipofectamin (Invitrogen). MGL positive cells were sorted using the MoFlo (DAKOcytomation, Glostrup, Denmark). The extracellular part of MGL (amino acids 61-289) was amplified on pRc/CMV-MGL with PCR, confirmed by sequence analysis and fused at the C-terminus to human IgG1-Fc in the Sig-pIgG1-Fc-vector. MGL-Fc was produced by transient transfection of CHO cells. MGL-Fc concentrations were determined by ELISA.

Glycan array (Consortium for Functional Glycomics)

Biotinylated synthetic or natural glycan structures were coated at saturating densities to streptavidin coated high binding capacity black plates (Pierce, Rockford, IL) and probed with MGL-Fc (2.5 μg/ml). Bound MGL-Fc was detected using a FITC-labeled anti-human IgG-Fc antibody. Plates were read at 485-535 nm on a Wallac Victor^2 1420 multi-label counter (Perkin Elmer, Wellesley, MA). Standard procedures for glycan array testing are available at (http://www.functionalglycomics.org). The repertoire of glycan structures probed are described elsewhere[23,24].

MGL-Fc adhesion assay

S. mansoni soluble egg antigens and biotinylated polyacrylamide-coupled glycoconjugates were coated (5 μg/ml or as indicated) on streptavidin coated plates (Pierce) or NUNC maxisorb plates (Roskilde, Denmark) overnight at room temperature. Plates were blocked with 1% BSA and MGL-Fc was added (1 μg/ml) for 2 hours at room temperature in the presence or absence of 10 mM EGTA or 20 μg/ml mAbs. Binding was detected using a peroxidase labeled anti-human IgG-Fc antibody

(Jackson, West grove, PA). To identify the carbohydrate nature of the MGL ligands, NUNC maxisorb plates were coated with goat anti-human Fc antibody (4 μg/ml, Jackson), followed by a 1% BSA blocking step (30 minutes at 37°C) and MGL-Fc (1 μg/ml for 1 hour at 37°C). MGL-Fc coated plates were incubated overnight at 4°C with tumor cell lysates (10 x 10^6 cells/ml). After extensive washing 1 μg/ml biotinylated or peroxidase labeled lectins (Sigma) were added for 2 hours at room temperature. Binding of biotinylated lectins was detected using peroxidase-labeled streptavidin (Vector Laboratories, Burlingham, CA).

Flow cytometry and cellular adhesion assays

Cells were incubated with primary antibody (5 μg/ml), followed by staining with a secondary FITC-labeled anti-mouse antibody (Zymed, San Francisco, CA) and analyzed on FACScalibur (BD Pharmingen, San Diego, CA). Streptavidin coated fluorescent beads (488/645 nm, Molecular Probes, Eugene, OR) were incubated with 1 μg of the PAA-coupled glycoconjugates or biotinylated soluble egg antigens. Fluorescent bead adhesion assay was performed as previously described[2] and analyzed on FACScalibur and presented as the percentage of cells which have bound the fluorescent beads. 96-well plates (NUNC maxisorb) were coated overnight at room temperature with biotinylated PAA-glycoconjugates (5 μg/ml) or SEA (2 μg/ml) and afterwards blocked with 1% BSA. Calceine AM labeled DCs (Molecular probes) were added for 1.5 hours at 37°C in the presence or absence of 10 mM EGTA or 10 μg/ml mAbs. Nonadherent cells were removed by gentle washing. Adherent cells were lysed and fluorescence was quantified on a Fluorstar spectrofluorimeter (BMG Labtech, Offenburg, Germany).

RESULTS

MGL specifically recognizes terminal α- or β-linked GalNAc residues that naturally occur as part of glycoproteins or glycosphingolipids

To allow efficient screening of multiple potential carbohydrate ligands, we constructed an MGL-Fc chimeric protein, with the extracellular portion of human MGL (amino acids 61-289) fused to a human IgG1-Fc tail. Recombinant MGL-Fc was produced in CHO cells and using an ELISA based method we show that MGL-Fc indeed comprises of the extracellular domains of MGL fused to the human IgG1-tail (Fig. 1A).

To identify the carbohydrate recognition profile and potential function of MGL, the MGL-Fc chimera was used to screen for carbohydrate ligands on the glycan array of the Consortium for Functional Glycomics[23,24]. MGL-Fc strongly bound the monosaccharides α-GalNAc and β-GalNAc, whereas no interaction was observed with the related sugar galactose, or other monosaccharides tested (Fig. 1B). The exclusive specificity of MGL for GalNAc was confirmed using α- and β-GalNAc monosaccharides multivalently linked to polyacrylamide (PAA) (Fig. 1C). Our results demonstrate that MGL exclusively recognizes GalNAc, both in α- or β-linked

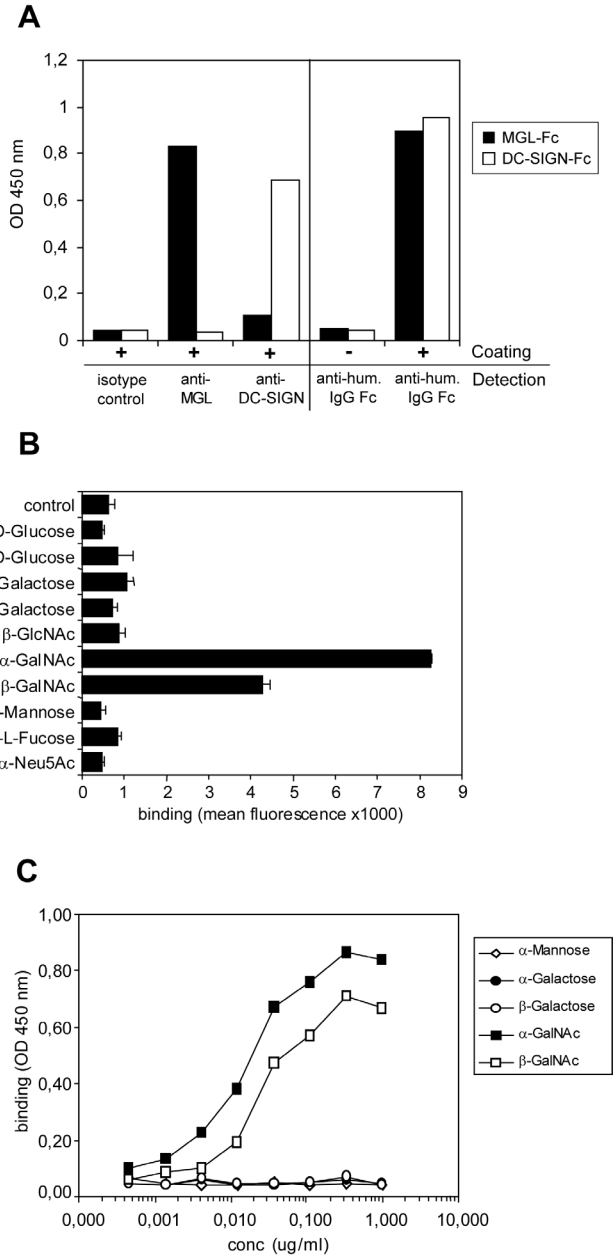

Figure 1. MGL specifically recognizes GalNAc residues. (A) MGL-Fc comprises of a human IgG1 tail and the extracellular domains of MGL. MGL-Fc was detected in ELISA by anti-MGL and anti-human IgG-Fc Abs. DC-SIGN-Fc was used as a control. (B) MGL interacts with GalNAc residues. MGL-Fc binding to monosaccharides coated on the glycan array was detected by FITC-labeled anti human Fc-antibody. (C) MGL recognizes multivalent GalNAc. Streptavidin plates were coated with biotinylated PAA-glycoconjugates. MGL-Fc binding was determined by ELISA. Standard deviation <0.02 OD 450 nm. One representative experiment out of three is shown.

configuration, whereas no specificity for either α- or β-Galactose was observed.
We next investigated whether MGL recognizes GalNAc moieties present in extended oligosaccharides. During O-glycan synthesis α-GalNAc is substituted with other monosaccharides to form several O-glycan core structures. Using the glycan array we investigated whether MGL recognizes specific O-glycan core structures (Fig. 2A). MGL specifically interacted with a single α-GalNAc residue, also known as the Tn antigen. Sialylation of the α-GalNAc residue completely abrogated MGL binding, whereas sulfation did not alter MGL reactivity. Substitution on position 3 of the α-GalNAc, as found in the core 1-4 structures, abrogated MGL binding. In contrast, addition of another α-GalNac-residue to position 3 (core 5) or a β-GlcNAc to position 6 (core 6) did not affect MGL binding activity (Fig. 2A). Thus, MGL-Fc specifically recognizes Tn antigen and core 5 and 6 O-glycan structures.
Glycan antigens with terminal β-GalNAc residues, such as the LDN glycan epitope

(LacdiNAc, GalNAcβ1-4GlcNAc) and its derivate LDNF (GalNAcβ1-4(Fucα1-3)GlcNAc) are found in humans, but are much more abundantly expressed by human helminth parasites such as *Schistosoma mansoni*[25,26]. In humans complex-type glycans usually contain a lactosamine unit (LacNAc, Galβ1-4GlcNAc) or Lewis X (Galβ1-4(Fucα1-3)GlcNAc). Indeed MGL recognized both LDN and LDNF oligosaccharides, but not their galactose-containing counterparts lactosamine or Lewis X (Fig. 2B).
Certain glycosphingolipids contain terminal GalNac residues, such as the gangliosides GM2, GD2 and the Forssman glycolipid (globopentosylceramide, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4GlcCer). MGL strongly interacted with the oligosaccharide component of GM2 and GD2, whereas no binding was observed to oligosaccharides that do not contain a terminal GalNAc, as found in GM3 and GD3 (Fig. 2C). In addition, MGL-Fc recognized the Forssman glycolipid in a glycolipid ELISA (data not shown).
Our results strongly support a MGL recognition profile of terminal α- and β-linked GalNAc residues (Table 1).

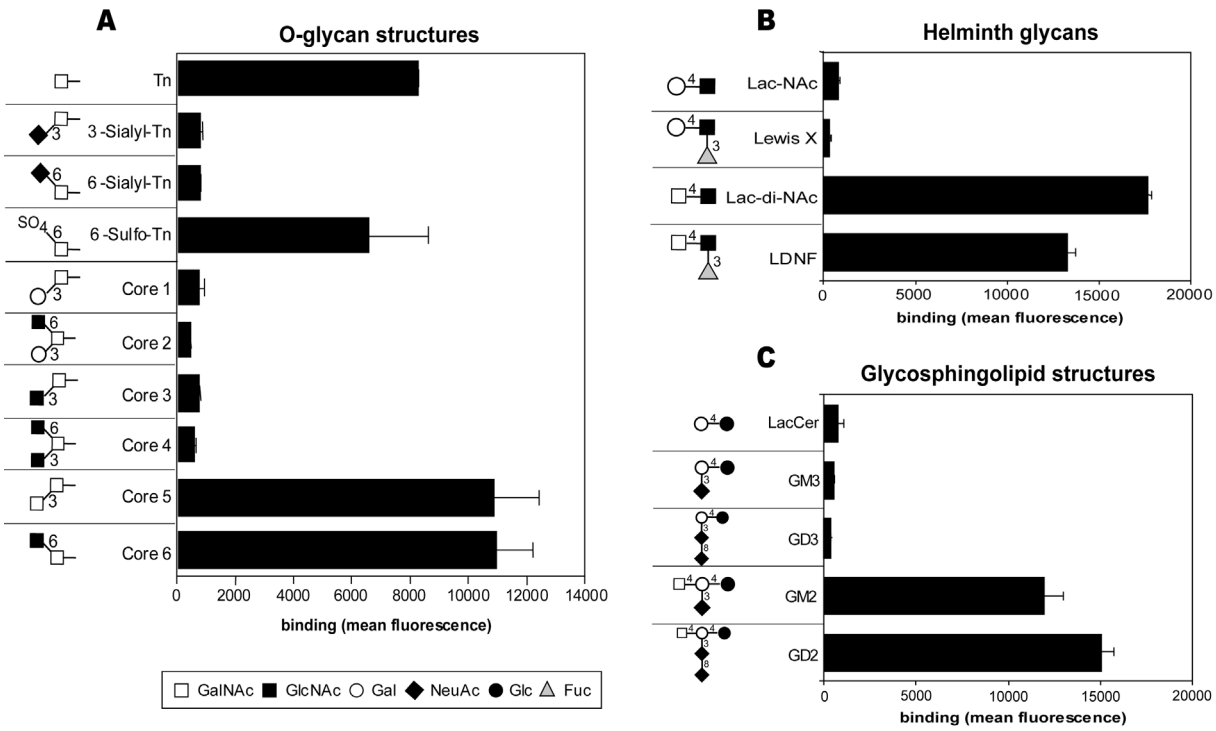

Figure 2. MGL recognizes terminal GalNAc residues in O-glycans, helminth associated glycans and glycans that are part of glycosphingolipids. MGL-Fc binding was determined by glycan array analysis of (A) O-glycan core structures, (B) LDN and LDNF glycans, and the related oligosaccharides LacNAc and Lewis X and (C) ganglioside glycan structures. Schematic representation of all carbohydrate structures tested is indicated in the figures.

MGL is the major GalNAc-receptor on human dendritic cells

To confirm the GalNAc-specificity of MGL-Fc, MGL-expressing cells were analyzed for their carbohydrate binding characteristics. CHO-MGL transfectants expressed

high levels of MGL on their cell surface (Fig. 3A). Both α-GalNAc and β-GalNAc-PAA coupled beads showed strong binding activity to CHO-MGL, but not to the parental CHO cells. MGL-mediated adhesion is completely inhibited by the Ca^{2+}-chelator EGTA or an anti-MGL antibody, but not by an isotype control antibody. Neither α- nor β-galactose was recognized by CHO-MGL (Fig. 3B), indicating that cell surface expressed MGL exhibits a similar GalNAc specificity as recombinant MGL-Fc.

Affinity	Glycan structure	Name	S/N ratio[a]
High	GalNAcα1-3Gal-	Adi	40.17
	GalNAcβ1-4GlcNAc-	LacdiNAc (LDN)	35.68
	Neu5Acα1-8Neu5Acα1-3(GalNAcβ1-4)Galβ1-4Glcβ-	GD2	30.35
	Bi-LDNF	Biantennary LDNF	28.97
	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ-	A (type 2)	27.50
	GalNAcβ1-4(Fucα1-3)GlcNAc-	Fucα3-LacdiNAc (LDNF)	26.75
	Neu5Acα1-3(GalNAcβ1-4)Galβ1-4Glcβ-	GM2	24.11
	GlcNAcβ1-6GalNAcα-	Core 6	22.06
	GalNAcα1-3GalNAcα-	Core 5	21.90
	α-GalNAc-	α-N-acetyl-D-galactosamine	16.62
	Bi-LDN	Biantennary LDN	13.32
	6-Sulfo-GalNAcα-	α-N-acetyl-D-galactosamine-6-sulfate	13.25
Medium	GalNAcα1-3(Fucα1-2)Galβ-	Atri-long	10.11
	β-GalNAc	β-N-acetyl-D-galactosamine	8.62
Low	Galβ1-4GlcNAcβ1-6GalNAcα-	6-LacNAc-Tn	6.57

Table 1. Overview of carbohydrate structures recognized by the C-type lectin MGL. [a] Signal to noise ratio. Signal ratios (S) were determined by dividing the fluorescence value obtained for each carbohydrate by the background fluorescence level (497 fluorescence units). All signal ratios were averaged out to a noise ratio (N) of 4.04. Individual signals (S) were then divided by 4.04 to give the listed signal to noise ratio (S/N). High affinity S/N>3*N; medium affinity S/N>2*N, low affinity S/N>1*N.

Human immature DCs naturally express MGL on the cell surface (Fig. 3A). Despite the fact that DCs express multiple C- type lectins, binding of α- or β-GalNAc to DCs was substantially blocked by anti-MGL antibodies and not by the anti-DC-SIGN antibody (Fig. 3C). DCs displayed only low binding to galactose, which could not be attributed to MGL, as the interaction could not be blocked by anti-MGL antibodies. DC-expressed MGL displayed an exclusive GalNAc-specificity and our results clearly indicate a major role for MGL in GalNAc recognition by DCs.

MGL functions as a pattern recognition receptor for helminth antigens

The specific recognition of LDN and LDNF by MGL (Fig. 2) prompted us to look at the interaction of MGL with helminth glycan antigens. The soluble egg antigens

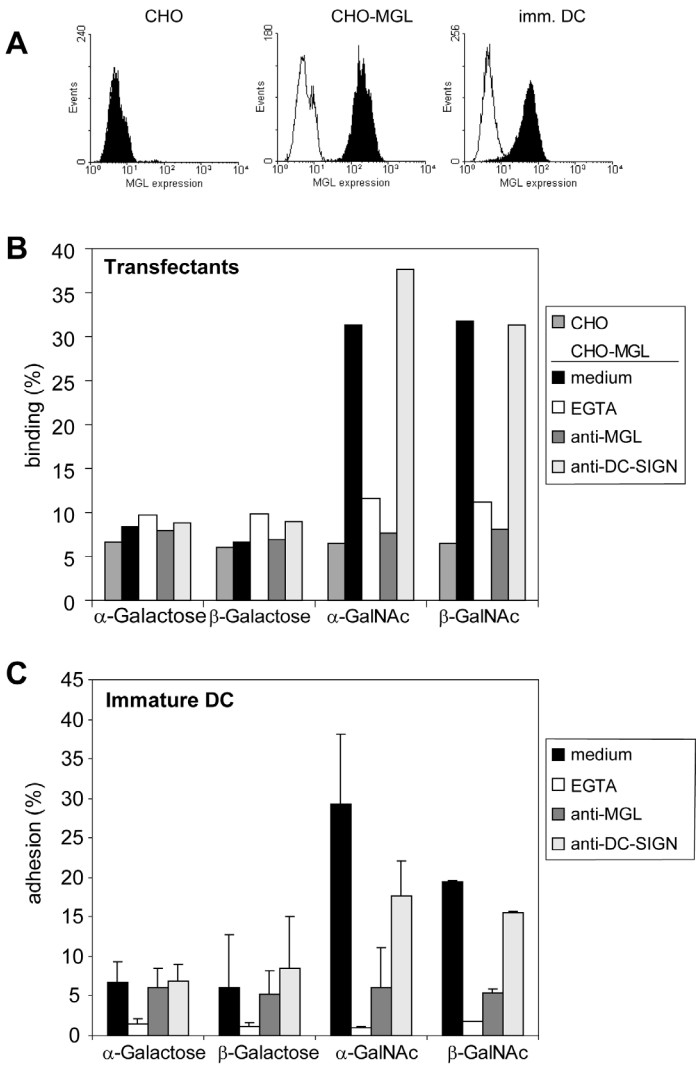

Figure 3. Both cellular and recombinant MGL have identical specificities for α- and β-linked GalNAc. (A) MGL expression on CHO transfectants and immature DCs. Open histograms represent the isotype control and filled histograms represent the anti-MGL mAb staining. (B) CHO-MGL strongly interacts with GalNAc but not with galactose in the bead adhesion assay. Specificity was determined in the presence of the Ca^{2+}-chelator EGTA, blocking antibodies to MGL or isotype control antibody (anti-DC-SIGN). Standard deviation for the bead adhesion assay is <5%. (C) MGL is the major GalNAc receptor on immature DCs. Binding of DCs to PAA-coupled carbohydrates was determined using plate adhesion assay in the presence or absence of EGTA, blocking antibodies to MGL or DC-SIGN. All results are representative for three independent experiments.

(SEA) of the human parasite *Schistosoma mansoni* were selected as a natural source for LDN and LDNF glycans. Both LDN-PAA and SEA-coupled beads showed high binding activity to CHO-MGL, but not to parental CHO cells (Fig. 4A). MGL-mediated adhesion was specific, as shown by the complete block with anti-MGL antibodies or EGTA, but not with an isotype control antibody.

Since SEA is composed of a several glycoproteins, monoclonal antibodies against carbohydrate epitopes were used to determine the relative contribution of LDN and LDNF in the binding of MGL to SEA (Fig. 4B). Binding of MGL to SEA was substantially blocked by anti-LDN mAbs (38% reduction) and to a lesser extent by anti-LDNF mAbs (22% reduction), whereas a combination of both anti-LDN and anti-LDNF mAbs further reduced MGL reactivity (46% inhibition), indicating that MGL recognizes both terminal β-GalNAc residues of LDN and LDNF structures present in SEA. Next, blocking antibodies were used to determine the relative contribution of MGL in relation to other DC expressed C-type lectins, in the binding of LDN and SEA. MGL mediated 50% of the adhesion of DCs to LDN, as shown by the significant reduction in binding with anti-MGL antibodies (Fig. 4C). Since other C-type lectins on DCs, such as DC-SIGN, are reported to be involved in binding SEA

through Lewis X structures[20], the interaction of DCs with SEA was further analyzed using blocking anti-MGL and anti-DC-SIGN antibodies. Both MGL and DC-SIGN are responsible for 30% of the DC reactivity, whereas a combination of anti-DC-SIGN and anti-MGL antibodies reduced adhesion by 50% (Fig. 4C). Therefore, DC-expressed MGL functions as a pattern recognition receptor for helminth parasites.

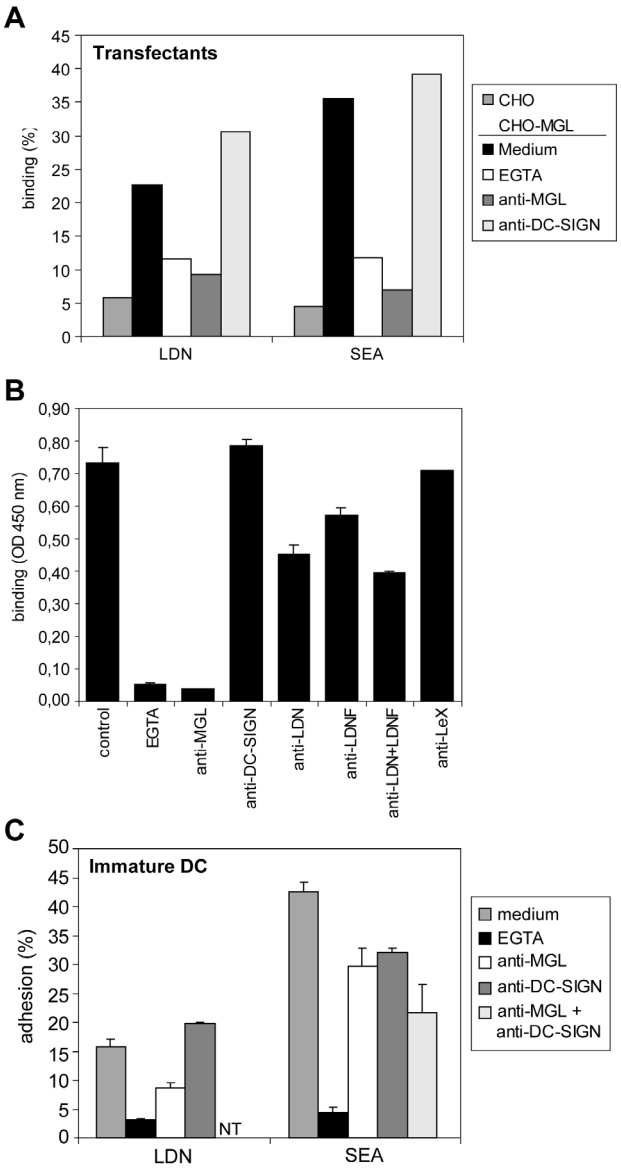

Figure 4. MGL strongly interacts with soluble egg glycoproteins of *Schistosoma mansoni*. (A) CHO-MGL binds to LDN glycans and SEA as measured by bead adhesion assay. Specificity was determined in the presence of the Ca^{2+}-chelator EGTA, blocking antibodies to MGL or isotype control antibody (anti-DC-SIGN). Standard deviation for the bead adhesion assay is <5%. (B) MGL interacts with LDN and LDNF glycans present in the SEA. Binding of MGL-Fc to SEA was determined by ELISA in the presence or absence of EGTA, blocking mAbs to MGL and DC-SIGN (isotype control) or specific anti-glycan mAbs. (C) Binding of SEA to immature DCs is mediated by both MGL and DC-SIGN. Binding of DCs was determined by plate adhesion assay in the presence or absence of EGTA, blocking mAbs to MGL or DC-SIGN. NT, not tested. All results are representative for three independent experiments.

The tumor specific Tn antigen is bound with high affinity by MGL

Tumorigenicity is associated with an increased degree of sialylation and a reduction in length of the O-glycans expressed[27]. Tumor cells, especially of adenocarcinoma origin, are frequently positive for the Tn antigen (single α-GalNAc linked to serine or threonine). The tumor-associated Tn antigen was preferentially recognized by MGL (Fig. 2A); therefore several adenocarcinoma cell lines and melanoma cell lines were analyzed for specific recognition by MGL. All adenocarcinoma cell lines tested and 3 out of 6 melanoma cell lines displayed a strong interaction with MGL-Fc (Fig. 5A).
One high binding adenocarcinoma cell line, ZR75-1, and one melanoma cell line,

00.09, were selected to investigate the nature of the recognized carbohydrates on tumor glycoproteins. Carbohydrate ligands were captured from tumor cell lysates with MGL-Fc and probed with commercially available plant/invertebrate lectins with known specificities. The lectins, SBA and HPA, which both have specificity for α-GalNAc residues, showed consistent reaction with glycoproteins, captured by MGL from tumor cells (Fig. 5B), indicating the presence of terminal α-GalNAc residues on the tumor antigens recognized by MGL.
Since our previous data showed that recombinant and cellular MGL have identical carbohydrate recognition profiles, these results indicate that MGL might function as a DC-specific receptor for tumor derived cell types.

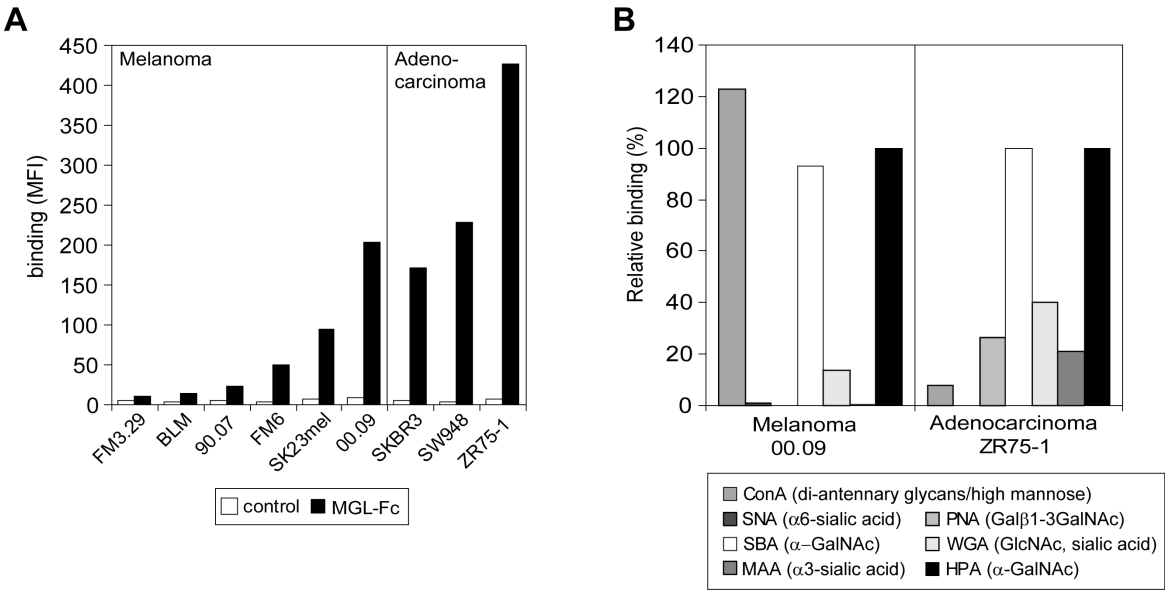

Figure 5. MGL recognizes α-GalNAc residues on tumor cells. (A) MGL recognizes both adenocarcinoma and melanoma tumor cells. Binding of MGL-Fc to tumor cell lines was determined by flow cytometry. (B) MGL recognizes GalNAc residues on tumor glycoproteins. Presence of specific carbohydrate structures on glycoproteins captured by MGL was determined using biotinylated plant/invertebrate lectins with known specificities in an ELISA based assay. Values were normalized to the HPA reactivity. Standard deviation <0.02 OD 450 nm. All results are representative for three independent experiments.

DISCUSSION

Here we report the carbohydrate recognition profile of the C-type lectin MGL and the implications these results may have for the recognition of self-gangliosides, helminth parasites and tumor cells by DCs.
Using a high throughput glycan array screening, developed by the Consortium for Functional Glycomics, terminal α-and β-linked GalNAc residues were identified as the main carbohydrate determinants for MGL recognition. Carbohydrate microarrays for studying protein-carbohydrate interactions are just emerging and most of them

work as a proof-of-principle, using lectins with known specificities to validate the array system[17]. To our knowledge this is one of the first reports describing the identification of the carbohydrate recognition profile of a single C-type lectin with the use of a carbohydrate array. Carbohydrate recognition profiles of selectins, langerin and DC-SIGN homologues have been further refined with the use of glycan arrays [17-19].

Both galactose/GalNAc and GalNAc specificity have been reported for human MGL[12,16]. Initially the specificity of MGL was evaluated using lysates of MGL transfected into COS-1. Purification on a galactose-Sepharose column showed that galactose, GalNAc and even fucose were able to elute MGL from the purification column[12]. However subsequent binding studies with recombinant MGL produced in a bacterial expression system identified a restricted GalNAc specificity[16]. Moreover, the MGL specificity was not confirmed for cells naturally expressing MGL. In this article we define the carbohydrate recognition profile of human MGL-Fc chimeric protein by glycan array and confirm this profile using MGL transfectants and MGL positive immature dendritic cells.

Our data clearly demonstrate that both recombinant MGL-Fc and MGL expressed by transfectants and DCs have an identical and exclusive specificity for terminal α- or β-linked GalNAc residues and no specificity for galactose. Substitution on position 3 or 4 of the GalNAc residue and sialylation, either on position 3 or 6 of the GalNAc, completely eliminates MGL recognition. The effect of the sialylation could be due to the addition of a dominant negative charge, which has been described for other C-type lectins to interfere in binding[22]. However addition of a negative charge in the form of a sulfate group on position 6 does not affect MGL binding (Fig. 2A). The blocking effect of sialic acid on position 6 of the GalNAc is unlikely to be due to steric hindrance, since the addition of GlcNAc on this position does not preclude MGL recognition. The exclusive GalNAc specificity indicates that human MGL is functionally most closely related to mouse mMGL2, which shares the GalNAc specificity with human MGL[15].

Our finding that anti-MGL antibodies do not completely block binding of α-GalNAc or LDN to DCs, suggests that DCs express next to MGL another receptor with GalNAc specificity. No candidate C-type lectins have been reported to be expressed by immature DCs, since the well known galactose/GalNAc-specific lectin ASGP-R[28] abundantly expressed by liver parenchymal cells, is not expressed by DCs (data not shown). Although MGL and ASGP-R have partially overlapping carbohydrate recognition profiles (GalNAc and galactose/GalNAc respectively), the exclusive expression of MGL on immature DCs and Mϕs, implicates non-redundant cellular functions for these C-type lectins. Recently galectin-3 was shown to be involved in uptake of SEA by Mϕs through recognition of LDN[29]. Since galectin-3 is expressed by DCs[30], galectin-3 might be a promising candidate receptor. Recognition of LDN by DC could therefore be mediated by both MGL and galectin-3 simultaneously.

Most C-type lectins contain special motifs within their cytoplasmic tails, which facilitate antigen uptake and thereby enhance antigen processing and presentation.

Although C-type lectins do not directly stimulate the immune system, they affect the balance between immunity and tolerance by influencing Toll-like receptor signaling[8,31]. C-type lectins probably have an important endogenous role in maintaining tolerance towards self-glycoproteins[5].
The specific interaction of MGL with SEA glycoproteins containing LDN and LDNF, demonstrates that MGL functions as a pattern recognition receptor for the human helminth parasite *Schistosoma mansoni*. SEA are known to skew the immune system towards a Th2-type response[32]. Recently, Ebola and Marburg filoviruses have been reported as pathogenic ligands for human MGL[33]. Interestingly both SEA and filoviruses target DC-SIGN as well, in a fucose and high-mannose type manner respectively[20,34]. In addition, the mouse mannose receptor recognizes SEA in a mannose dependent fashion[35]. The residual binding of DC to SEA, after complete block of MGL and DC-SIGN, might therefore be mediated by the human mannose receptor. Since several pathogens misuse the tolerogenic pathway induced by C- type lectins for their own survival[36], it will be interesting to pursue how targeting of pathogens to MGL and other cooperating C-type lectins may modulate DC maturation and the induction of adaptive immune responses.
Furthermore, MGL recognized glycosphingolipids, mainly of the ganglioside subtype. As MGL has the capacity to internalize synthetic glycoconjugates[37], it might internalize glycolipids for loading onto CD1 molecules, similarly as reported for the C-type lectin langerin[38]. Langerin is capable of loading CD1a molecules with foreign glycolipids derived from *Mycobacteria leprae*, however the gangliosides which bind MGL are normally expressed in the spleen[39]. The fact that gangliosides inhibit APC function[40], may hint to a possible function of MGL in antigen presentation and maintenance of tolerance to self-glycolipids.
Our data demonstrate that MGL might be involved in the recognition of tumor cells by DCs. In normal leukocytes and epithelial cells O-glycans are essentially all of core 1 or extended core 2 subtype. Tumorigenicity is associated with increased sialylation and a reduction in length of the O-glycans expressed[27]. Tumor cells, especially of adenocarcinoma origin, are frequently positive for the Tn antigen (single α-GalNAc) or TF antigen (Galβ1-3GalNAc). Positivity for the Tn antigen-specific lectin HPA serves as a diagnostic marker for a wide range of adenocarcinomas and coloncarinomas and is associated with lymph node metastases, poor prognosis and lower survival rates[41]. A high correlation was found between the GalNAc-specific lectins HPA/SBA, and MGL recognition of α-GalNAc in tumor glycoproteins. Recently, cytotoxic T cells have been described with specificity for the Tn antigen[42], indicating that it is possible to generate a T cell response against carbohydrate antigens. Targeting of tumor antigens containing the Tn antigen to MGL on DCs can potentially result in enhanced presentation in MHC I and II. Yet targeting of antigens to C-type lectins without any additional DC activation may have tolerizing effects and inhibit anti-tumor responses[5,6]. We postulate that the expression of Tn antigen by tumors might modulate immune responses via targeting to C-type lectins, such as MGL.

Our data shows that MGL specifically recognizes terminal α- and β-linked GalNAc moieties that are present on tumor derived cell types, pathogens such as helminth parasites and self-antigens, such as glycosphingolipids. Future studies should address whether antigen targeting to MGL on APCs, such as DCs and Mϕs, leads to antigen presentation as well as immune modulation.

ACKNOWLEDGEMENTS

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CHAPTER 4

REGULATION OF EFFECTOR T CELLS BY ANTIGEN PRESENTING CELLS VIA INTERACTION OF THE C-TYPE LECTIN MGL WITH CD45

Sandra J. van Vliet, Sonja I. Gringhuis, Teunis B.H. Geijtenbeek and Yvette van Kooyk

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ABSTRACT

Homeostatic control of T cells involves tight regulation of T effector cells to prevent excessive activation that can cause tissue damage and autoimmunity. Little is known, however, about whether antigen presenting cells (APCs) are also involved in maintaining immune system homeostasis once effector T cells are stimulated. Here we found that immature APCs downregulated effector T cell function by a mechanism involving the C-type lectin MGL expressed by APCs. Glycosylation-dependent interactions of MGL with CD45 on effector T cells negatively regulated T cell receptor-mediated signaling and T cell-dependent cytokine responses, which in turn decreased T cell proliferation and increased T cell death. Thus, regulation of effector T cells by MGL expressed on APCs may provide a target for regulating chronic inflammatory and autoimmune diseases.

INTRODUCTION

Professional antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages (Mϕs) line all peripheral tissues, where they 'screen' their environments for pathogens or changes in immune homeostasis[1]. APCs have a unique plasticity: they are capable of adopting various functional phenotypes depending on the environmental conditions and the external stimuli they receive. They are instrumental in initiating adaptive immunity and pathogen clearance; however, they also actively control several processes involved in tissue repair, clearance of apoptotic cells and maintenance of tolerance to nonharmful self-antigens[2,3]. Microbial products such as lipopolysaccharide induce immune activation by triggering DC maturation, which is required for naive T cell activation and for the differentiation of classically activated Mϕs to a state of enhanced ability to kill intracellular pathogens. In contrast, glucocorticoids 'instruct' the differentiation of tolerogenic DCs[4] or 'alternatively activated' Mϕs (aaMϕs) with 'durable immaturity' and a reduced capacity to stimulate naive T cell proliferation. Those properties link tolerogenic DCs and aaMϕs to the maintenance of steady state conditions and in the protection from excessive inflammatory responses[5].

All APC subsets express exclusive sets of C-type lectin receptors (CLRs) that recognize specific carbohydrate moieties in a calcium-dependent way[6]. CLRs recognize and internalize both exogenous and endogenous glycoproteins or lipids[7] for presentation on major histocompatibility complex (MHC) class I or II or CD1 molecules[8]. However, targeting of antigen to CLRs, without a concomitant 'danger signal' results in immune senescence and T cell unresponsiveness[9]. Several CLRs, therefore, may be important in controlling immune homeostasis by clearing self-antigens and thereby actively induce tolerance. In contrast, at least one CLR, dectin-1, mediates signaling that acts in synergy with pathways induced by Toll-like receptor 2, which indicates that some CLRs may also have immune-activating properties[10].

The CLR 'Mϕ galactose-type lectin' (MGL) is expressed exclusively by myeloid APCs

such as human immature DCs and aaMϕs[11,12]. The carbohydrate-recognition profile of human MGL consists of a unique specificity for terminal N-acetylgalactosamine (GalNAc) residues[13]. The mouse homologues of MGL have distinct carbohydrate recognition profiles[14], indicative of different functions for MGL in mice and humans.
We set out to define potential counter-receptors (glycoproteins that carry the carbohydrate ligand) for human MGL on immune cells and identified GalNAc epitopes present on CD45 of effector T cells (T_{eff} cells). Although other receptors for CD45 have been described, MGL is the first cellular receptor to our knowledge to be identified on 'professional' APCs that interacts with CD45. CD45 is the prototypic tyrosine phosphatase expressed on T cells[15]. CD45 dephosphorylates the negative regulatory tyrosine residue (Y505) on the protein tyrosine kinase Lck, resulting in active Lck and T cell receptor (TCR) signaling. CD45 is alternatively spliced into five different isoforms with different glycosylation[16,17], which changes during T cell differentiation and activation[18,19]. Thus, cell-specific glycosylation of CD45 could provide a mechanism for influencing various immunological pathways, including TCR and cytokine receptor signaling.
Here we demonstrate that DCs or aaMϕs cultured in dexamethasone, with low expression of costimulatory molecules, suppressed proliferation of $CD4^+$ T_{eff} cells through interaction of MGL with CD45 expressed by T cells. That reduced CD45 phosphatase activity and inhibited Lck activation and calcium mobilization. MGL-mediated downregulation of T_{eff} cell activation was also characterized by reduced production of inflammatory cytokines and induction of T cell death. Our results demonstrate an important function for CLRs on APCs in the regulation of T cell homeostasis and the silencing potentially harmful T cell activation.

MATERIALS AND METHODS

Cells and cell lines

All cell lines were maintained in RPMI medium containing 10% FCS (Invitrogen) or Iscove's medium containing 5% FCS. Immature DCs were cultured for 4-7 days from monocytes obtained from buffy coats of healthy donors (Sanquin) in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml). DCs were matured by addition of 1 μg/ml lipopolysaccharide (*Salmonella typhi*, Sigma-Aldrich). Mϕs were generated from monocytes by culture in the presence of 1000 U/ml GM-CSF. Dexamethasone was added at a concentration of 1 μM.

Antibodies and reagents

The following monoclonal antibodies were used: anti-DC-SIGN[20] (AZN-D1; IgG1), anti-CD11a (SPV-L7 and NKI-L15); anti-CD43 (MEM-59); anti-CD45 (MEM-28; provided by V. Horesji, Academy of Sciences of the Czech Republic, Prague, Czech Republic); anti-CD45 (D3/9), anti-CD45RA (RP1/11), anti-CD45RB (RP2/21), and anti-CD45RC (RP1/12; all provided by F. Sánchez-Madrid, Hospital Universitario de la Princesa, Madrid, Spain[17]); anti-CD27 ($GF4^{bio}$; provided by R. van Lier, Academic

Medical Center, Amsterdam, The Netherlands); stimulating anti-CD3 (T3b[50]); anti-CD80, anti-CD86 and anti-CD45RO (UCHL-1; all from BD Biosciences); phycoerythrin-conjugated CD4 (clone 13B8.2,) and phycoerythrin-conjugated CD8 (clone B9.11; all from Beckman Coulter); mouse anti-mouse H2-D^b (28-14-8); anti-CD1a (OKT6); anti-CD68 (EMB11; Dako,); anti-CD163 (Edhu1); anti-mannose receptor (3.29.B1); anti-CD11b (Bear-1); and anti-CD11c (SHCL-3). Antibodies 18E4 and 1G6.6 (both IgG2a) were generated by immunization of Balb/c mice with purified MGL-Fc. Hybridoma supernatants were screened for the presence of anti-MGL on CHO-MGL transfectants. MGL-Fc and control Fc-proteins (ICAM-3-Fc or CAMEL-Fc) were generated as described[13].

Flow cytometry and MGL-Fc binding assays

For surface expression analysis, cells were incubated with primary antibody (5 μg/ml), followed by staining with a secondary FITC-labeled anti-mouse (Zymed, San Francisco, CA) and analyzed on a FACScalibur (BD Biosciences). For analysis of MGL ligand expression, cells were incubated with MGL-Fc (10 μg/ml) in a solution of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% ($^w/_v$) BSA, followed by staining with a secondary FITC-labeled anti-human Fc (Jackson) and analyzed on a FACScalibur. T cells were identified by costaining for 10 min at 21°C with subset-specific markers.

Immunohistochemistry

Cryosections of healthy tissues (7 μm in thickness) were fixed with 100% aceton and stained with primary antibodies (10 μg/ml) for 1 hour at 37°C. Sections were counterstained with isotype-specific Alexa fluor 488- or Alexa fluor 594-labeled anti-mouse (Molecular probes). Rheumatoid arthritis synovial biopsies were collected after informed consent was obtained from patients with active rheumatoid arthritis who were undergoing arthroscopy.

Cellular adhesion assays

CHO and CHO-MGL cells were seeded at a density of 35×10^3 cells per well in 96-well plates. Cells labeled with calcein AM (Molecular probes) were added for various times at 37°C. Nonadherent cells were removed by gentle washing. Adherent cells were lysed and fluorescence was quantified on a Fluorstar spectrofluorimeter (BMG Labtech).

Immunoprecipitation and immunoblot analysis

Cell surfaces were biotinylated for 30 min at 4°C with 0.5 mg/ml sulfo-NHS-biotin (Pierce) in PBS and cells were then lysed for 1 hour at 4°C in lysisbuffer (10 mM triethanolamine, pH 8.2, 150 mM NaCl, 1 mM $MgCl_2$, 1mM $CaCl_2$, 1% ($^v/_v$) Triton X-100, containing EDTA-free protease inhibitors (Roche Diagnotics)). MGL ligands were immunoprecipitated with Fc-protein-coupled protein A-sepharose beads (CL-4B; Pharmacia). Immunoprecipitation products were seperated by SDS-PAGE and

were transferred to nitrocellulose membranes. Blots were blocked with BSA, followed by immunoblot analysis with streptavidin-coupled peroxidase (Vector Laboratories) or specific antibodies, then secondary peroxidase-conjugated goat anti-mouse (Jackson). Blots were developed by enhanced chemiluminescence.

MGL-Fc ligand ELISA

NUNC maxisorb plates were coated for 1 hour at 37°C with goat anti-human Fc (4 μg/ml; Jackson), followed by a blocking step with 1% ($^{w}/_{v}$) BSA and then incubation for 1 hour at 37°C with MGL-Fc (1 μg/ml). Plates coated with MGL-Fc were incubated overnight at 4°C with cell lysates (10×10^6 cells/ml for cell lines, 500×10^6/ml for PBL). After extensive washing 1 μg/ml of monoclonal antibodies was added for 2 hours at 21°C. Binding was detected with peroxidase-labeled goat anti-mouse (Jackson).

CD45 phosphatase activity, Lck phosphorylation and calcium mobilization

Jurkat cells were stimulated for 20 min at 37°C on CHO or CHO-MGL cells, then were washed with ice-cold PBS and were lysed in Ph lysis buffer (20 mM HEPES, pH 7.2, 2 mM EDTA, 2 mM DTT, 1% $(^{v}/_{v})$ Nonidet P-40, 10% $(^{v}/_{v})$ glycerol containing protease inhibitors). Cellular debris and nuclear material were removed by centrifugation at 20,000*g* for 15 min at 4°C. CD45 was immunoprecipitated with MEM-28 and protein A-sepharose-beads. CD45 phosphastase activity was quantified by incubation of the beads for 4 hours at 37°C with 2 mM 4-nitrophenyl phosphate (Roche) in CD45 Ph assay buffer (100 mM HEPES, pH 7.2, 2 mM EDTA, 2 mM DTT). The resulting color change was assessed at 405 nm. Specificity was determined by the addition of a specific CD45 phosphatase inhibitor (Calbiochem). Serum-starved Jurkat cells were incubated for indicated time points with MGL-Fc or control-Fc and phosphorylation of Lck Y505 was measured by flow cytometry with an Alexa Fluor 488-labeled antibody specific for the phosphorylated form of Lck Y505 (BD Biosciences) according to the manufacturer's instructions. As a positive control, a sample of 5 mM H_2O_2 (15 min 37°C) was included. Jurkat cells were 'loaded' with fluo-3 AM (Molecular probes), prewarmed at 37°C and were analyzed by flow cytometry. Cells were stimulated with MGL-Fc-coated beads or anti-CD3 (5 μg/ml).

Cell death assay

Jurkat cells were seeded in 24-well plates at a density of 200×10^3 cells per well in a final volume of 500 μl and were cultured overnight with MGL-Fc or control-Fc (10 μg/ml) with or without soluble anti-CD3 (2.5 μg/ml). Free GalNAc (50 mM) was added to block MGL function. Cellular apoptosis was determined on basis of forward-scatter and side-scatter patterns and staining with Annexin V-FITC (BD Biosciences) and/or 7-AAD (Molecular probes).

T cell proliferation and cytokine production

PBLs (100×10^3) were seeded onto CHO or CHO-MGL cells and then stimulated for

24 hours with soluble anti-CD3 or were seeded onto plates coated with MGL-Fc (5 μg/ml) and anti-CD3 (1 μg/ml) or with control-Fc and anti-CD3, followed by 24 hours of culture. Cytokine expression in the supernatants was determined by ELISA according to the manufacturer's protocol (Biosource international). Purified $CD4^+$ T cells (100×10^3; more than 97% pure) were cultured for 4 days at 37°C with allogeneic APC at various ratios. T cell proliferation was assessed by incorporation of [methyl-^{3}H]Thymidine (Amersham biosciences).

Statistical analysis

Student's t-test was used for statistical analysis. Results with a P value of less than 0.05 were considered significant.

RESULTS

MGL is expressed on APCs in vivo and in vitro

We cultured various APC subsets from human monocytes and analyzed their expression of MGL. We used the CLR 'DC-SIGN' as a well known marker of DCs

APC subset	Isotype control (MFI)	MGL expression (MFI)
Mϕs	10	**81**
Alternatively activated Mϕs (Dexamethasone)	14	**122**
Innate activated Mϕs (LPS)	21	20
Classically activated Mϕs (LPS/IFNγ)	71	85
Immature DCs	6	**28**
Immature DCs (IL-10)	5	**25**
Immature DCs (Dexamethasone)	10	**81**
Mature DCs (LPS)	7	9
Mature DCs (Poly I:C)	3	5
Mature DCs (Flagellin)	3	4

Table 1. MGL expression on cultured APCs. Different APC subsets were cultured from freshly isolated human monocytes in the presence of indicated cytokines. The following concentrations were used: for Mϕ culture: GM-CSF 1000 U/ml, dexamethasone 10^{-6} M, LPS 1 μg/ml, IFNγ 1000 U/ml and for DC culture: GM-CSF 500 U/ml, IL-4 800 U/ml, dexamethasone 10^{-6} M, LPS 1 μg/ml, Poly I:C 10 μg/ml and Flagellin 5 μg/ml. MGL expression was determined by flow cytometry using the 18E4 antibody. One representative experiment out of two is shown. MFI, mean fluorescent intensity.

and aaMϕs[20,21]. We detected MGL on immature DCs and Mϕs; its expression both as protein and mRNA was enhanced on immature DCs and aaMϕs generated in the presence of dexamethasone (Fig. 1A and data not shown). Lipopolysaccharide-induced DC maturation completely abolished MGL expression, and we did not detect MGL expression on other mature DCs or activated Mϕs (Table 1). As APCs cultured *in vitro*, except immature Mϕs, expressed MGL and DC-SIGN together, we assessed whether MGL and DC-SIGN were present on similar APC subsets *in vivo*. In healthy lymph nodes and skin, MGL and DC-SIGN were expressed on separate APC subsets in the outer zones of the paracortex and dermis respectively, whereas in the jejunum, there was a complete overlap of MGL and DC-SIGN expression (Fig. 1B). Characterization of the MGL$^+$ cells in skin and lymph node identified them as myeloid-type APCs (Table 2).

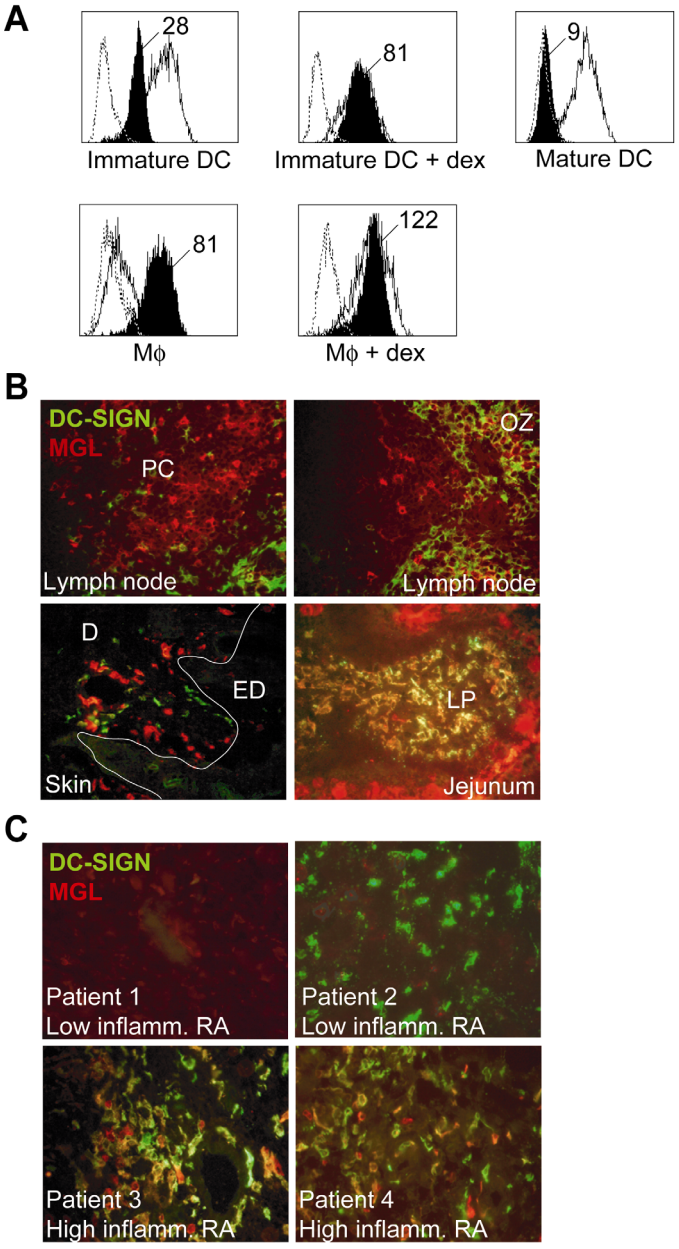

Figure 1. MGL expression in tissues and on APCs generated in vitro. (A) Flow cytometry of MGL and DC-SIGN expression on monocyte-derived APCs. Dotted lines, isotype control staining; solid lines, DC-SIGN expression; filled histograms, MGL expression. Numbers above peaks indicate mean fluorescent intensity for MGL. Dex, dexamethasone. Results are representative for three independent experiments. (B) Fluorescence microscopy of human tissue sections of jejunum, lymph node (lung draining) and skin, stained with anti-MGL (red) and anti-DC-SIGN (green). The red staining surrounding the jejunum lamina propria is non-specific, due to background staining of the mucus layer. PC, paracortex; OZ, outer zone paracortex in proximity of the medullary and paracortical sinuses; D, dermis; ED, epidermis; LP, lamina propria. (C) Fluorescence microscopy of human tissue biopsies obtained from four patients (1-4) with rheumatoid arthritis (RA), stained with anti-MGL (red) and anti-DC-SIGN (green). Original magnification (B, C) x400.

To assess MGL expression *in situ* at sites of high numbers of infiltrating T cells, such as in chronic inflammatory conditions, we obtained synovial biopsies from four patients with rheumatoid arthritis and stained them for MGL and DC-SIGN. We classified biopsies as 'low inflammatory rheumatoid arthritis with a diffuse infiltrate' or 'high inflammatory rheumatoid arthritis with many leukocytes infiltrating the tissue'. In the 'low inflammatory rheumatoid arthritis' biopsy samples, we detected no MGL expression throughout the whole biopsy sample, whereas in the 'high inflammatory rheumatoid arthritis' biopsy samples, we found abundant MGL and DC-SIGN single- and double-positive cells with a bright CLR expression (Fig. 1C). All rheumatoid arthritis biopsy samples had many infiltrating Mϕs (data not shown). These results collectively show that MGL is 'preferentially' expressed on dexamethasone-treated DC and Mϕ subsets as well as on APCs in several human tissues. MGL expression seems to coincide with chronic inflammatory conditions.

Marker	Skin	Lymph node
CD1a	+	–
CD68	+/–	+/–
CD163	–	–
DC-SIGN	–	–
Mannose receptor	+	+
CD11b	+	+
CD11c	+	+
CD3	NT	–

Table 2. Characterization of MGL$^+$ APC in skin and lymph node. Cryosections of human skin and lymph node (7 μm) were fixed with 100% aceton and stained with anti-MGL (10 ug/ml) and primary antibodies to the markers listed (5 μg/ml) for 1 hour at 37°C. Sections were counterstained with isotype-specific Alexa fluor 488- or Alexa fluor 594-labeled anti-mouse antibodies. The following antibodies to CD1a (OKT6), CD68 (EMB11), CD163 (Edhu1), DC-SIGN (AZN-D1), Mannose receptor (3.29.B1), CD11b (Bear-1), CD11c (SHCL-3) and CD3 (T3b) were used. Listed is the co-expression of markers on the MGL$^+$ cells: –, negative; +, positive; +/–, subpopulation of the MGL$^+$ cells co-express the marker; NT, not tested. One donor tissue out of two is shown. MGL thus represents a marker for the skin APCs described in Angel *et al* JI 2006.

CD45 on human T cells is a counter-receptor for MGL

To identify self-ligands of MGL, we screened a panel of human hematopoietic cell lines for MGL binding. MGL-Fc is a chimeric molecule consisting of the extracellular domains of MGL fused to the human IgG1 Fc-tail. We found that MGL-Fc strongly interacted with the erythroleukemic cell line K562 and with the T cell lines H9, HSB-2 and Jurkat (Fig. 2A and table 3). MGL binding was inhibited by addition of the calcium chelator EGTA, free GalNAc monosaccharides or by antibody to MGL (anti-MGL), confirming the specificity of the interaction (Fig. 2A). To verify T cell binding, we isolated primary human PBMCs and tested their binding to MGL-Fc. MGL-Fc specifically bound PBLs, as shown by complete inhibition of MGL-Fc binding with EGTA (Fig. 2B). In addition to T cells, natural killer cell and B cell subpopulations

bound MGL-Fc, whereas monocytes did not (data not shown). To confirm the interaction of MGL and T cells at a cellular level, we did cell-cell adhesion experiments assessing the adhesion of PBLs and Jurkat cells to MGL-transfectants. PBLs specifically adhered to CHO cells expressing MGL ('CHO-MGL cells') compared with the background binding to parental CHO cells. Also, Jurkat cells firmly adhered to CHO-MGL cells, whereas there was only background adhesion to the parental CHO cells (Fig. 2C).

To identify the MGL 'counter-receptors', we immunoprecipitated cell surface-biotinylated Jurkat lysates with MGL-Fc. Immunoblot analysis of precipitated proteins identified three membrane proteins of approximately 220, 190 and 110 kDa

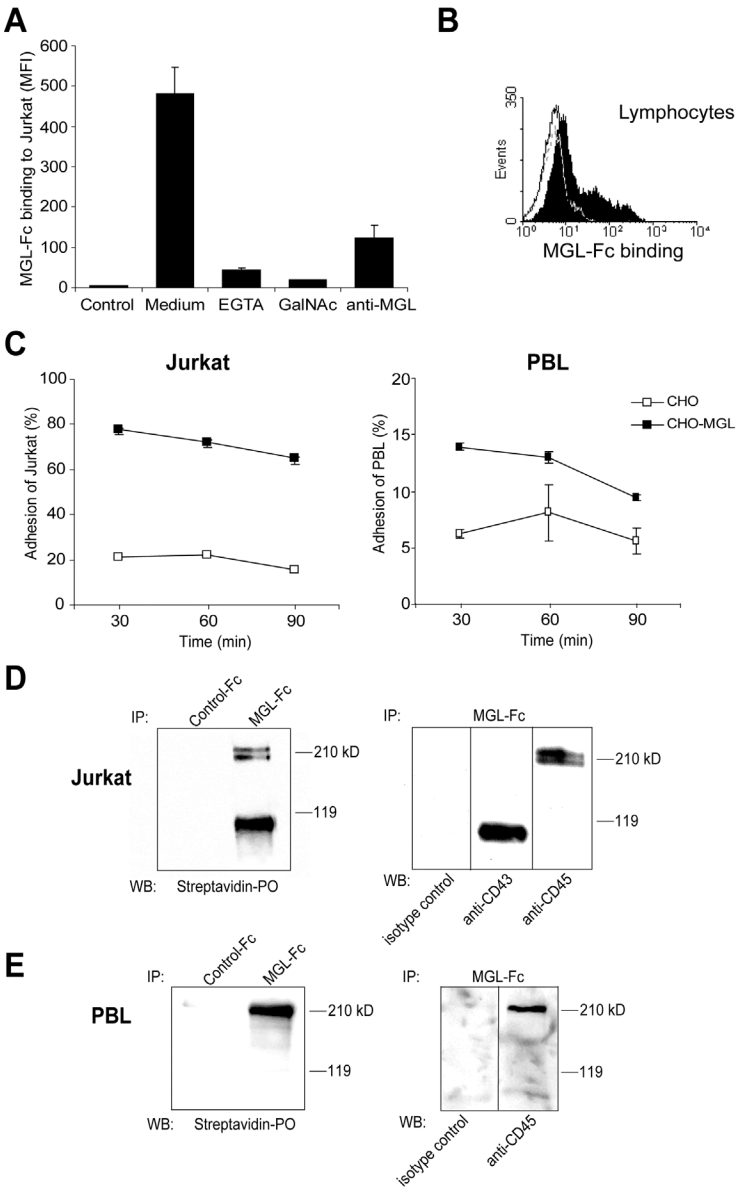

Figure 2. MGL binds CD45 on human lymphocytes. (A, B) Flow cytometry of Jurkat T cells and PBLs. (A) Binding of MGL-Fc with relative high affinity, inhibited by the addition of EGTA (10 mM), free GalNAc (100 mM) or anti-MGL (20 μg/ml). Control, no MGL-Fc added. (B) Binding of MGL-Fc to human PBLs. Dashed line, control-Fc; filled histogram, MGL-Fc; solid line, MGL-Fc plus 10 mM EGTA. Data are from one representative donor of twenty. (C) Better adherence of Jurkat T cells (left) and human PBLs (right) to CHO-MGL cells than to parental CHO cells. (D) Immunoprecipitation and immunoblot of lysates of surface-biotinylated Jurkat cells with MGL-Fc or control-Fc and streptavidin-peroxidase (left) or with monoclonal anti-CD43, anti-CD45 or anti-LFA-1 (isotype control, right). (E) Immuno-precipitation and immunoblot of lysates of surface-biotinylated human PBLs with MGL-Fc or control-Fc and streptavidin-peroxidase (left) or with monoclonal anti-CD45 or anti-LFA-1 (isotype control, right). All results are representative of at least three independent experiments (error bars, s.d.).

Name	Cell type	MGL-Fc recognition	MGL counter-receptors
K562	Erythroleukemic cell line	++	CD45
HSB-2	Acute lymphoblastic leukemia	++	CD43, CD45
H9	Cutaneous T cell lymphoma	+	CD45
Jurkat E6.1	T cell leukemia line	+++	CD43, CD45
Peer	Acute lymphoblastic leukemia	–	NA
Raji	Burkitt's lymphoma	–	NA
MonoMac-6	Monocyte-macrophage cell line	–	NA
U937	Histiocytic lymphoma	–	NA

Table 3. MGL-Fc recognition of hematopoietic cell lines. Cell lines were examined for MGL recognition by staining with MGL-Fc. Counter-receptors were determined by MGL-Fc ligand ELISA. –, no binding; +, low MGL-Fc binding (MFI <100); ++, intermediate MGL-Fc binding (MFI 100-1000); +++, high MGL-Fc binding (MFI >1000); NA, not applicable.

(Fig. 2D). Based on the observed molecular weights and heavy O-glycosylation, we considered the mucin-type proteins CD43 (95-115 kDa) and CD45 (180-220 kDa) as primary candidates for the MGL counter-receptors. Immunoprecipitation of MGL ligands with MGL-Fc and immunoblot analysis with specific antibodies demonstrated that the upper two bands corresponded to two CD45 isoforms and the lower 110 kDa band to CD43 (Fig. 2D). After a similar procedure with cell surface biotinylation and immunoprecipitation with MGL-Fc, we identified only one main

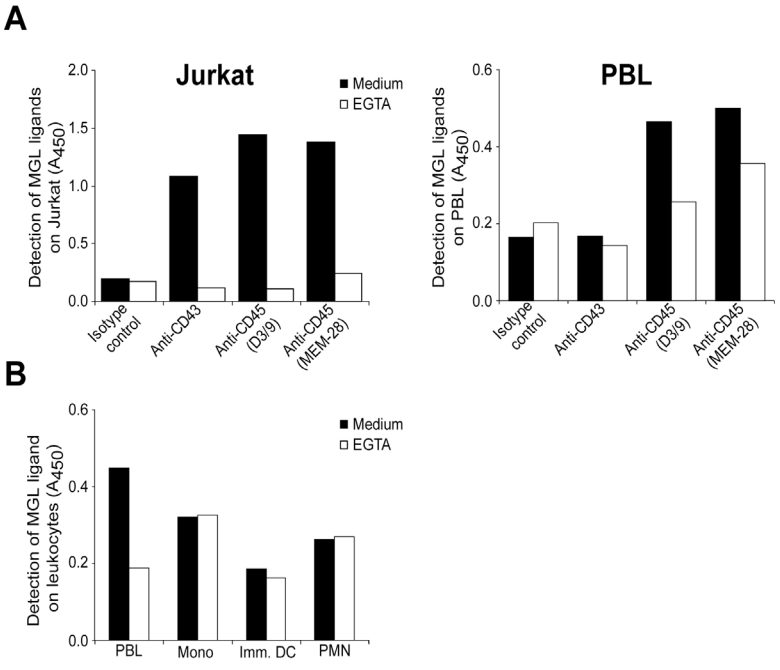

Figure 3. CD45 is a counter-receptor for MGL on lymphocytes. (A) Capture of ligands from PBL or Jurkat lysates on MGL-Fc coated plates detected by specific antibodies to CD43, CD45 or LFA-1 (isotype control) in an MGL-Fc ligand ELISA. MGL-Fc binding was blocked by the addition of 10 mM EGTA. One representative experiment out of three is shown. Standard deviation <0.05 A_{450}. (B) MGL-binding proteins captured from lysates of PBLs, monocytes, immature DCs or PMNs by MGL-Fc coated plates and detected by specific antibodies to CD45 in an MGL-Fc ligand ELISA. MGL-Fc binding was blocked by the addition of 10 mM EGTA. One representative experiment is shown. Standard deviation <0.05 A_{450}.

MGL ligand of approximately 200 kDa in PBL lysates. To determine whether the immunoprecipitated band corresponded to CD45, we stripped the blot and reprobed with anti-CD45 or control antibodies. Immunoblot analysis with anti-CD45 identified the band as CD45 (Fig. 2E). We did not note binding of MGL to CD43 on PBLs, which we concluded was probably because of the high content of sialic acid on lymphocyte CD43[22], which would inhibit recognition of GalNAc structures by MGL[13].
To confirm those results, we 'captured' MGL ligands from lysates by use of plates coated with MGL-Fc. MGL-Fc specifically interacted with both CD43 and CD45 from Jurkat cells, as demonstrated by complete inhibition of the interactions with EGTA, whereas on PBLs, only CD45 was recognized (Fig. 3A). On all other cell lines studied that interacted strongly with MGL, CD45 was the main counter-receptor for MGL (Table 3). MGL exclusively recognized CD45 from lymphocytes and not from other leukocytes such as monocytes, immature DCs and polymorphonuclear neutrophils (Fig. 3B).

			Antibody	staining			MGL-Fc
30019 cells	**Isotype**	**Pan-CD45**	**CD45RA**	**CD45RB**	**CD45RC**	**CD45RO**	**binding**
Mock	–	–	–	–	–	–	–
CD45ABC	–	+	+	+	+	–	++
CD45AB	–	+	+	+	–	–	++
CD45BC	–	+	–	+	+	–	++
CD45B	–	+	–	+	–	–	++
CD45RO	–	+	–	–	–	+	–

Table 4. MGL recognition of CD45 isoforms. Expression of CD45 isoforms by 30019 transfectants, assessed by flow cytometry with antibodies specific for the A, B or C domain (left six cloumns of data). –, no staining; +, positive staining. The CD45 isoforms were precipitated with a 'pan-CD45' antibody and were 'probed' with MGL-Fc (far right column). –, no binding; ++, high binding (A_{450}>0.25). Specificity was determined in the presence of EGTA (data not shown).

Cd45 mRNA undergoes alternative splicing in its A, B and C domains, giving rise to five different isoforms on human leukocytes (CD45ABC, AB, BC, B and RO)[15,23]. The different CD45 isoforms can be distinguished on the basis of their reactivity with monoclonal antibodies specific for CD45RA, CD45RB or CD45RC. None of the antibodies directed against the protein backbone blocked the interaction of MGL with specific glycan structures on CD45 (data not shown). To investigate the recognition of different CD45 isoforms by MGL, we used single human CD45 isoform transfectants of the mouse cell line 30019[17]. Flow cytometry confirmed expression of the individual CD45 isoforms in the transfectants (Table 4). To analyze binding to MGL, we used an ELISA-based assay, in which the human CD45 isoforms were 'precipitated' from lysates of the various transfectants with a 'pan-CD45' antibody followed by a specific detection step with MGL-Fc. MGL-Fc bound all A-, B- or C-containing isoforms, whereas CD45RO was not recognized by MGL, although it was efficiently 'captured' from the lysate by the 'pan-CD45' antibody

(Table 4 and data not shown). We confirmed those results for PBLs by 'capturing' MGL ligands from PBL lysates and probing these with isoform-specific antibodies to CD45; again there was no binding between MGL and CD45RO, whereas we detected interactions of MGL with A, B or C containing isoforms (Table 5). Thus, all CD45 isoforms except CD45RO on human lymphocytes are counter-receptors for MGL. Expression of GalNAc epitopes on the individual CD45 isoforms constitutes a prerequisite for binding, a process regulated by T cell differentiation.

Capture	Detection					
	Isotype	**panCD45**	**CD45RA**	**CD45RB**	**CD45RC**	**CD45RO**
MGL-Fc	–	++	+	++	++	–

Table 5. MGL-Fc recognition of CD45 isoforms on PBLs. MGL counter-receptors were 'captured' from PBL lysates and 'probed' with antibodies specific for CD45 isoforms: –, no binding; +, low binding (A_{450}<0.25); ++, high binding (A_{450}>0.25). Specificity was determined in the presence of EGTA (data not shown).

MGL modulates CD45 activity and TCR signaling

CD45 affects cellular responses by controlling the threshold of sensitivity to external stimuli, especially those received by the TCR. Therefore, we investigated whether the MGL-CD45 interaction influenced CD45 phosphatase activity and function. We used the Jurkat E6.1 cell line, often used to study TCR-mediated signaling[24]. CD45 phosphatase activity can be regulated by the formation of dimers; increased formation of dimers results in decreased activity[25]. To maximize the formation of receptor dimers, we seeded Jurkat cells on CHO or CHO-MGL cells. We then lysed the Jurkat cells and measured CD45 phosphatase activity. Binding of MGL to CD45 resulted in a time-dependent decrease in CD45 phosphatase activity (Fig. 4A). There was no residual phosphatase activity in the presence of CD45-specifc phosphatase inhibitors, confirming the finding that the phosphatase activity was completely mediated by CD45.

The first 'downstream' target of CD45 in T cells is Lck. CD45 dephosphorylates the negative regulatory tyrosine residue Y505, resulting in an active pool of Lck. Reduced CD45 phosphatase activity after MGL-CD45 interaction could thus lead to decreased Lck activity through enhanced phosphorylation of Y505. In fact, we noted an increase in the phosphorylation status of Lck Y505 10-20 minutes after we added MGL-Fc to Jurkat cells; in this assay MGL-Fc funtions as an agonist (Fig. 4B). Blocking MGL function with free GalNAc lowered Y505 phosphorylation to control and unstimulated amounts.

Engagement of the TCR leads to activation of downstream signaling pathways, including intracellular calcium mobilization, which is critical in early T cell signal transduction. We labeled Jurkat E6.1 cells with the calcium sensitive dye fluo-3 AM and stimulated the cells with MGL-Fc coated beads and a stimulating antibody to CD3. For each sample we used the fluorescent beads to distinguish cells that

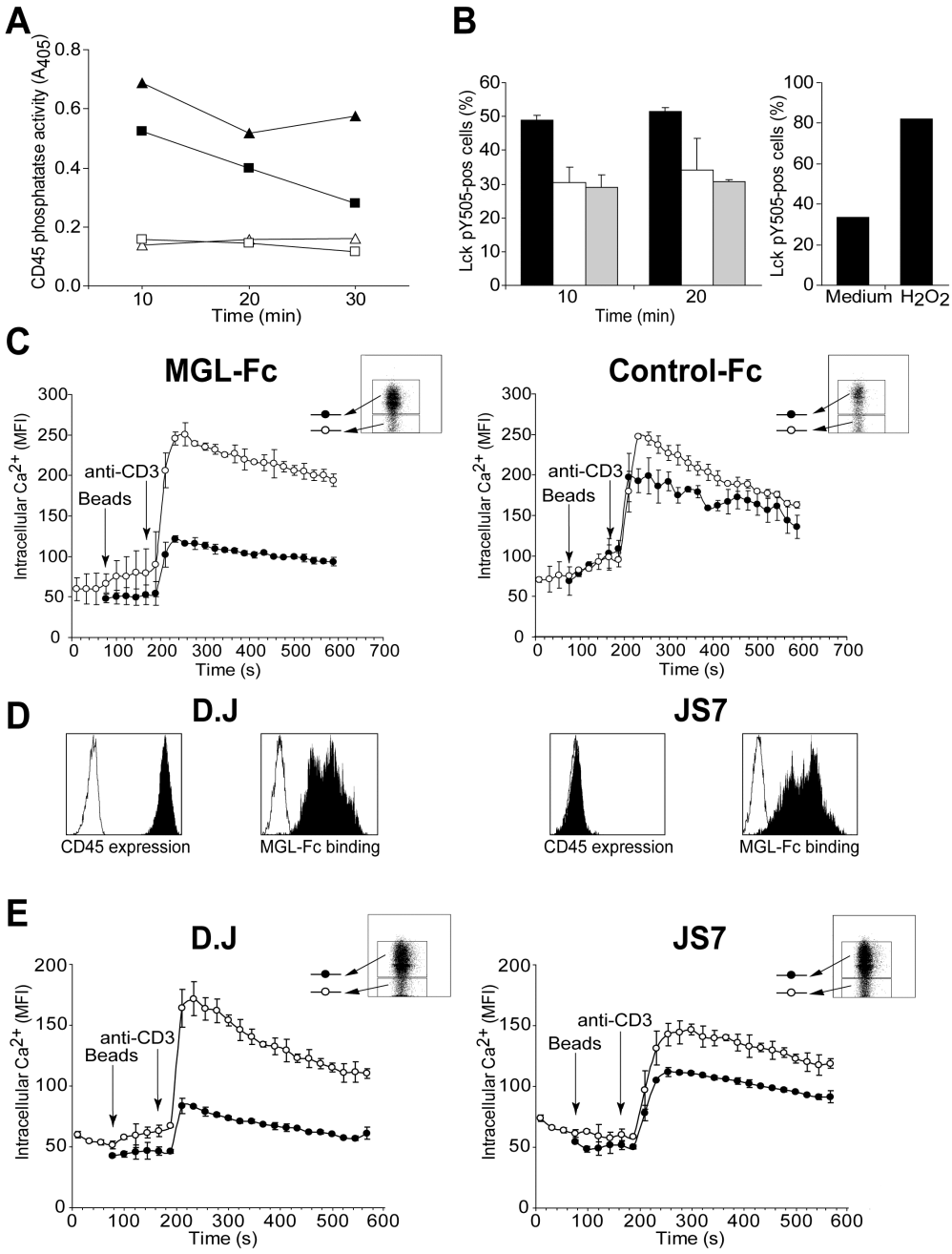

Figure 4. Binding of MGL downregulates CD45 phosphatase activity and TCR mediated calcium mobilization. (A) CD45-specific phosphatase activity in Jurkat T cells stimulated on CHO or CHO-MGL cells in the presence or absence of a specific CD45 phosphatase inhibitor. ▲ CHO, △ CHO plus CD45 inhibitor, ■ CHO-MGL, □ CHO-MGL plus CD45 inhibitor A_{405}, absorbance at 405 nm. s.d. < $0.05A_{405}$. (B) Flow cytometry of increased phosphorylation of Lck Y505 (Lck $pY505^{+}$; left panel) on Jurkat cells treated with MGL-Fc (filled bars) or MGL-Fc plus GalNAc (open bars). Grey bars, control-Fc. Right, unstimulated cells (medium) and cells incubated with 5 mM H_2O_2 (positive control). (C) Calcium mobilization, assessed by flow cytometry of fluo-3 AM-loaded Jurkat E6.1 cells after the addition of MGL-Fc beads (left) or control-Fc beads (right). (D) Flow cytometry of CD45 expression and MGL-Fc binding on the parental cell line D.J (left) and its $CD45^{-}$ clone JS7 (right). (E) Calcium mobilization, assessed by flow cytometry of fluo-3 AM-loaded D.J cells (left) and JS7 cells (right). Arrows in graphs (C, E) indicate the addition of beads and anti-CD3; Inserts are dot plots of bead adhesion (○, nonbinding cells; ● cells binding beads). MFI, mean fluorescent intensity. All results are representative for at least three independent experiments.

underwent MGL-mediated crosslinking from those that did not. Binding of MGL-Fc beads alone did not trigger calcium mobilization, although the subsequent calcium flux triggered by anti-CD3 was strongly downregulated. We did not find inhibition in Jurkat cells not treated with MGL-Fc beads or in Jurkat cells incubated with 'control-Fc' beads (beads coated with the control protein ICAM-3-Fc or CAMEL-Fc; Fig. 4C). To verify that the effects were mediated by CD45, we used the CD45⁻ Jurkat cell-derived JS7 cell line and the corresponding parental D.J cell line[26]. In contrast to D.J cells, JS7 cells completely lacked expression of CD45 (Fig. 4D), yet both cell lines had similar expression of CD3 and CD43 (data not shown). Both cell lines bound MGL-Fc with equal affinity; although on JS7 cells all MGL-Fc binding was mediated by CD43 (Fig. 4D and data not shown). Calcium mobilization triggered by anti-CD3 was inhibited in MGL-Fc-bound D.J cells; in contrast, in JS7 cells, calcium flux was lowered only marginally, probably because of the residual CD43-MGL interaction and the fact that CD43 can modulate TCR-mediated responses[19]. Those data confirmed that the reduction in calcium mobilization depended on the MGL-CD45 interaction (Fig. 4E). Thus, MGL can directly modulate CD45 activity and thereby negatively influence TCR-mediated signaling pathways.

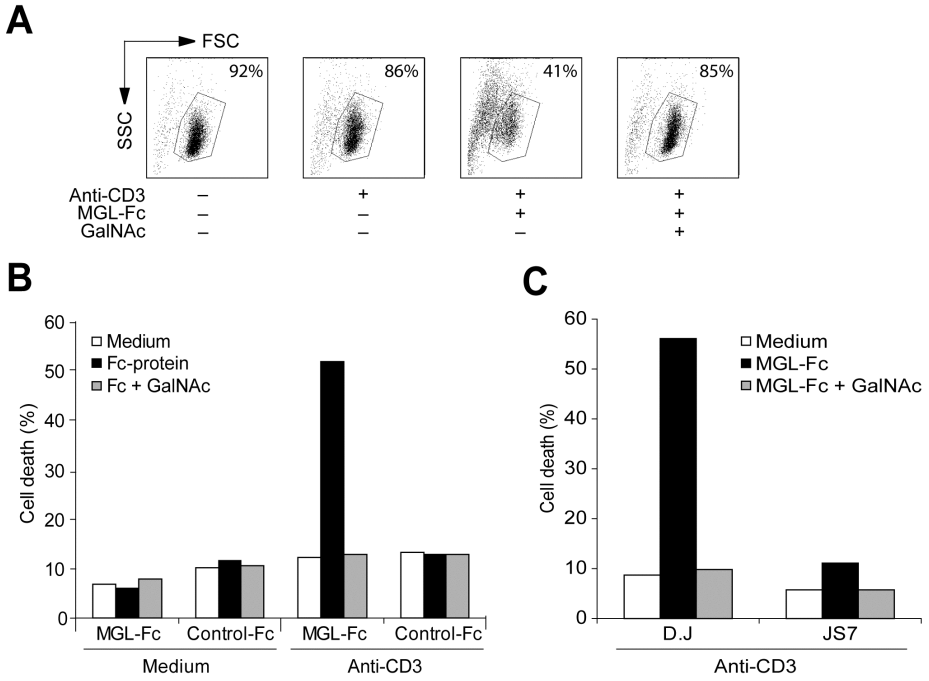

Figure 5. MGL induces CD45-dependent cell death. (A) Flow cytometry of Jurkat cells alone or after treatment with MGL-Fc; MGL-Fc and stimulating anti-CD3; or MGL-Fc, anti-CD3 and GalNAc. Numbers beside outlined areas indicate the percentage of cells in the live-cell gate. FSC, forward scatter; SSC, side scatter. (B, C) Quantification of cell death by flow cytometry with Annexin V-FITC and/or 7-amino-actinomycin D staining. (B) Jurkat cells treated with MGL-Fc or control-Fc in the presence (anti-CD3) or absence (medium) of anti-CD3 triggering. (C) Anti-CD3-stimulated CD45⁺ D.J and CD45⁻ JS7 cells incubated with MGL-Fc or MGL-Fc plus GalNAc. s.d. <5%. All results are representative for at least three independent experiments.

MGL-Fc induces TCR- and CD45-dependent cell death

We evaluated other effects of MGL-CD45 interaction on T cells. We observed that prolonged stimulation of Jurkat with anti-CD3 and MGL-Fc-induced crosslinking of CD45 led to enhanced cell death, as shown by flow cytometric analysis (Fig. 5A). MGL-Fc–anti-CD3 induced cell death resulted in cell surface exposure of phosphatidylserine, as measured by staining with annexin V (Fig. 5B), and nuclear fragmentation (data not shown). Cell death was completely dependent on combined CD3 triggering and MGL binding, as incubation with either anti-CD3 or MGL-Fc alone had little to no effect. We observed occasional cell death induced by MGL-Fc alone; however such cell death was always markedly increased by TCR stimulation (data not shown). The observed apoptosis was MGL-specific as addition of free GalNAc blocked cell death, while control Fc-protein did not induce cell death (Fig. 5B). In contrast to D.J cells, cell death could not be triggered in JS7 cells by MGL-Fc–anti-CD3, validating CD45-dependence for MGL-Fc–induced apoptosis (Fig. 5C).

MGL modulates T cell function

To evaluate whether the MGL-CD45 interaction affected primary T cell activation, we investigated the recognition of MGL of different human T cell subsets in the $CD4^+$ and $CD8^+$ compartment. Naive, memory and effector T cells can be distinguished by their differential expression of CD45RA and CD27[27]. Whereas naive T cells express both CD27 and CD45RA, memory T cells are $CD27^+CD45RA^-$. Memory T cells are characterized by their expression of CD45RO, although they retain expression of the CD45B isoform[17] (data not shown). T_{eff} cells generally lose expression of CD27; however, some T_{eff} cells regain expression of CD45RA. MGL showed a strong 'preference' for both $CD27^-CD4^+$ and $CD27^-CD8^+$ T_{eff} cells (Fig. 6A). MGL-Fc binding was specific as shown by inhibition in the presence of EGTA.

To study the effect of MGL binding on T cell activation, we analyzed the proliferative and cytokine responses of human lymphocytes. We seeded PBLs on CHO or CHO-MGL cells and then stimulated the cells with anti-CD3. After 24 hours, we collected supernatants and analyzed cytokine production. Production of both TNFα and IFNγ was much lower in the CD3-stimulated PBL-CHO-MGL coculture than in the PBL-CHO coculture (Fig. 6B). PBLs stimulated on plates coated with MGL-Fc and anti-CD3 produced less IFNγ and TNFα than did PBLs stimulated with control-Fc and anti-CD3 (Fig. 6C). This effect was completely mediated by MGL, as antibodies blocking MGL restored cytokine production to control amounts. In both systems, production of IL-6 production was not substantially affected, whereas IL-4, IL-5 or IL-10 could not be detected (data not shown). TNFα production was similarly reduced by MGL-Fc and anti-CD3 when we assayed purified $CD4^+$ or $CD8^+$ T cells instead of unfractionated PBLs (Fig. 7).

If MGL has a downregulatory effect on T cell activation and proliferation, blocking antibodies to MGL should reverse those effects and enhance proliferation. We cultured monocyte-derived APCs with high or low expression of MGL (Fig. 1A) together with highly purified allogeneic $CD4^+$ T cells in the presence or absence of

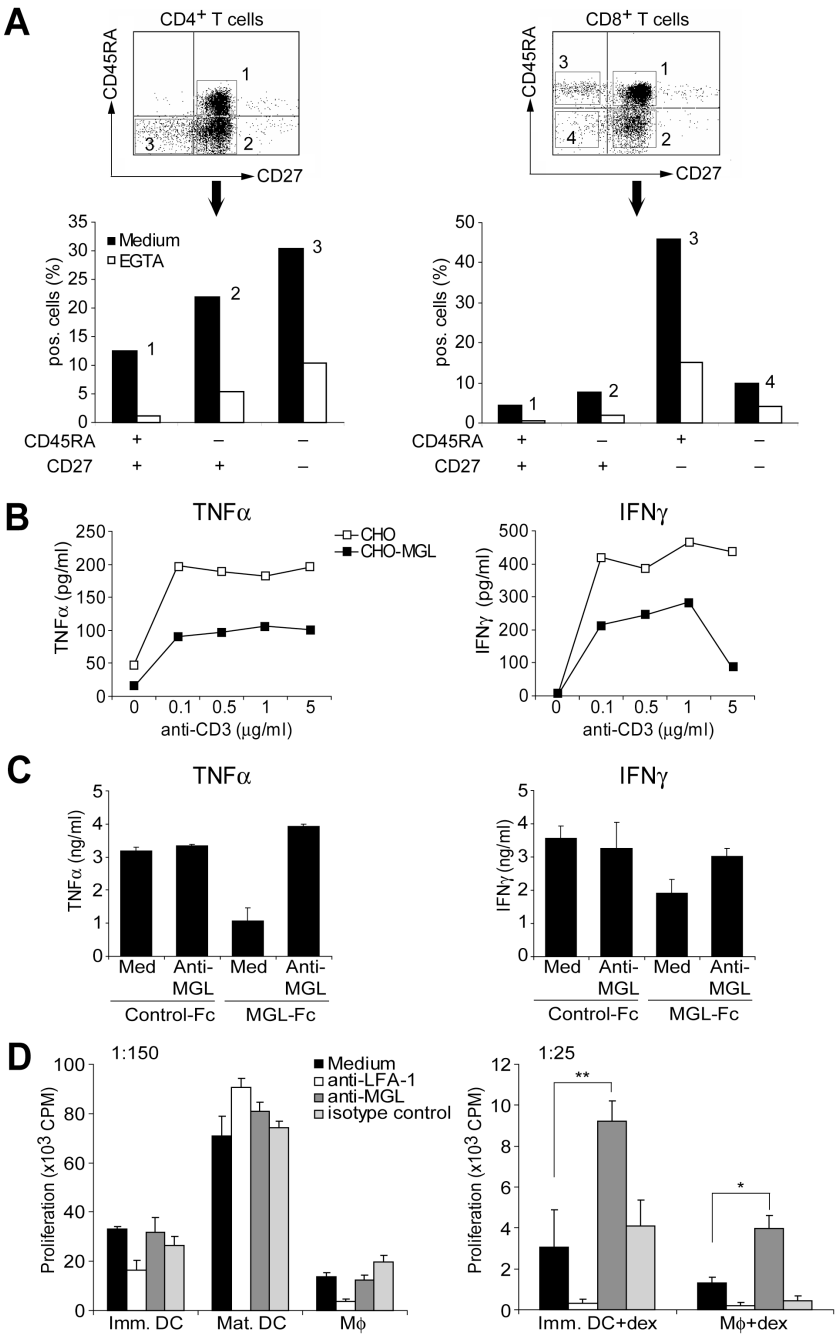

Figure 6. MGL on dexamethasone-treated APCs provides negative regulatory signals to human T_{eff} cells via CD45. (A) Flow cytometry of human T_{eff} cells stained for CD4 or CD8 and with antibodies specific for CD27 and CD45RA (top); cell populations in numbered gates were incubated with MGL-Fc with or without EGTA, followed by quantification of MGL-Fc-binding cells (bottom). s.d. <5%. (B,C) ELISA of TNFα (left) and IFNγ (right) in supernatants of human PBLs seeded for 24 hours on CHO or CHO-MGL cells and stimulated with soluble anti-CD3 (B) or on plates coated with MGL-Fc and anti-CD3 or control-Fc and anti-CD3 (C). Med, medium. (D) Partial reversion of the suppressive activity of dexamethasone-cultured APCs by anti-MGL. APCs and allogeneic purified $CD4^+$ T cells (more than 97% pure) were cultured together for 4 days at various ratios (above graphs) in the presence or absence of blocking anti-LFA-1 or anti-MGL. Isotype control, 28-14-8. Proliferation was assessed by incorporation of [methyl-^{3}H]Thymidine. Imm., immature; Mat., mature. **, P<0.01; *, P<0.05. All results are representative for three independent experiments.

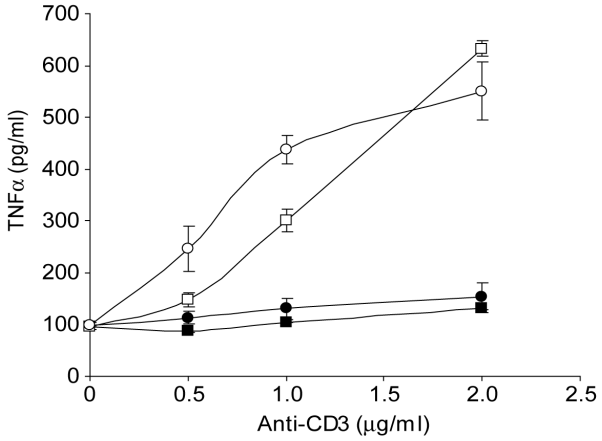

Figure 7. MGL binding reduces TNFα production by CD4⁺ and CD8⁺ T cells. Purified CD4⁺ and CD8⁺ T cells (more than 95% pure) were seeded on plates coated with MGL-Fc and anti-CD3 or control-Fc and anti-CD3. After 24 hours supernatants were harvested and TNFα production was measured by ELISA. ■ MGL-Fc (CD4⁺ T cells), □ Control-Fc (CD4⁺ T cells), ● MGL-Fc (CD8⁺ T cells), ○ Control-Fc (CD8⁺ T cells)

blocking antibodies to MGL and after 4 days assessed proliferation by incorporation of [methyl-^{3}H]-thymidine. The different APC subsets could be distinguished in immunogenic MGL$^{lo\text{-}neg}$ APCs (immature DCs, mature DCs and Mϕs), capable of inducing substantial T cell proliferation and in dexamethasone-treated MGL^{hi} APCs, which lack the ability to stimulate substantial T cell division, even at high APC/T cell ratios[28] (Fig. 6D). Anti-MGL had no effect on proliferation and cytokine production in the immunogenic APC-T cell cocultures (Fig. 6D and data not shown). In contrast, anti-MGL enhanced proliferation about threefold in the dexamethasone-treated APC-T cell cocultures (Fig. 6D), an effect not obtained for two isotype-matched control antibodies: a blocking antibody to the integrin LFA-1 and a non-binding isotype control (mouse anti-mouse H2-D^{b}). T cell proliferation by immature DCs was not enhanced by anti-MGL, in contrast to results obtained with the dexamethasone-treated DCs. That result was probably due to the higher expression of costimulatory molecules on the immature DCs (Table 6), which 'over-rides' any downregulatory effect of MGL. We did not detect any cytokines in the supernatants of the dexamethasone-treated APC-T cell cocultures. Those results indicated that blockade of MGL function eliminated the inhibitory effect of MGL on CD45 and enhanced T cell activation. Thus, MGL expressed by immature APCs with a tolerogenic phenotype specifically interacts with CD45 on T$_{eff}$ cells and thereby suppressing T cell activation.

APC subset	Isotype (MFI)	CD80 (MFI)	CD83 (MFI)	CD86 (MFI)
Immature DCs	6	31	52	238
Immature DCs + dex	25	29	26	81
Mature DCs	6	395	188	786
Macrophages	6	29	9	160
Macrophages + dex	20	21	20	49

Table 6. Expression of costimulatory molecules on cultured APCs. APC subsets were cultured from freshly isolated human monocytes in the presence of GM-CSF 500 U/ml/IL-4 800 U/ml (DCs) or GM-CSF 1000 U/ml (Mϕs). Dexamethasone was added at 10^{-6} M and LPS at 1 μg/ml. Expression of co-stimulatory molecules was determined by flow cytometry using antibodies to CD80, CD86 and CD83.

DISCUSSION

Here we have identified the CLR MGL as a receptor expressed by 'professional' APCs that binds to CD45 phosphatase on T cells. The mucin-type protein CD45 contains substantial O-linked carbohydrates, mainly localized in the differentially expressed A, B, and C domains[15]. MGL recognized all A-, B- and C-containing CD45 isoforms but not CD45RO. $CD45RA^{-}$ memory and effector T cells retain expression of the CD45B and CD45RO isoforms. As CD45RO was not recognized, MGL-mediated T cell binding is most likely mediated via the B isoform. The carbohydrate recognition profile of MGL has been elucidated; MGL has an exclusive specificity for terminal GalNAc structures[13]. Seven percent of all O-linked carbohydrates on CD45 obtained from human PBMCs are composed of the α–GalNAc moiety, consistent with MGL recognition of certain glycoforms of CD45[16]. CD45RO has only two O-linked glycan structures, which is probably not sufficient to sustain MGL recognition. Using the α-GalNAc-specific snail lectin *Helix pomatia* agglutinin, we detected terminal α-GalNAc structures on CD45 precipitated by MGL from human T cell lysates (data not shown). On Jurkat T cells both CD45 and CD43 were recognized by MGL; Jurkat cells contain a mutation in the gene encoding the glycosyltransferase chaperone Cosmc, resulting in increased exposure of terminal α-GalNAc structures on O-linked glycoproteins, explaining the high-affinity binding of MGL[29]. In contrast, on human T cells, CD43 is heavily sialylated, which abrogates MGL recognition[22].

Glycosylation is a cell-specific process, depending not only on the cellular composition of glycosyltransferases and glycosidases but also on the activation and differentiation status of the cell[19]. Glycosylation can thus influence diverse aspects of the immune system, although single glycans can have highly specific functions. CD45 glycosylation changes during T cell development, pheripheral activation and aging[18,30]. Furthermore, different CD45 isoforms are glycosylated differently[16]. Therefore, differential glycosylation of CD45 provides a 'scaffold' for CLR-based modulation of TCR-mediated responses. Apparently CD45 on T_{eff} cells has the appropriate glycan structures, because of cell-specific glycosylation, to ensure MGL recognition. Two putative 'counter-receptors' for human CD45, galectin-1 and CD22, have been identified, although their possible functions in regulating CD45 are unclear[31,32]. Although there is some controversy regarding whether CD45 activity can be controlled by receptor-mediated mechanisms[15]; we have demonstrated here that MGL on APCs can modulate CD45 phosphatase activity and function.

'Professional' APCs express a wide variety of unique CLR repertoires[6]. We have shown here that the CLRs MGL and DC-SIGN were localized on different APCs in skin and lymph nodes, whereas in lamina propria of the jejunum they were expressed together on the same APCs. MGL is also expressed on *in vitro* monocyte-derived DCs and Mϕs, APCs cultured with dexamethasone showed an enhanced and prolonged MGL expression and mature DCs did not express surface MGL.

Most cell surface-expressed CLRs internalize self antigens for presentation in steady-state conditions without causing overt DC maturation, thereby actively inducing

tolerance toward self-antigens[7]. In contrast, crosslinking of the CLR Dectin-1 induces intracellular signaling pathways, leading to production of inflammatory mediators such as reactive oxygen species and TNFα[10]. Whereas CLR-mediated signaling can modulate APC function, MGL is involved in regulation of the responder T cell activity. We have shown that MGL 'preferentially' recognized antigen-experienced $CD27^{-}$ T_{eff} cells, which are characterized by copious proinflammatory cytokine production and tissue-homing properties[27]; in addition, T_{eff} cells do not require extensive costimulation, as antigen recognition is sufficient for activation of these cells[33]. T_{eff} cells are also present in large numbers in chronic inflammatory diseases and parasitic infections; in those conditions MGL expression often upregulated[11]. Upregulation of MGL expression during the course of rheumatoid arthritis, for example, could be a 'self-protecting' mechanism initiated to prevent excessive inflammation and tissue damage. Tolerogenic APCs with low costimulatory molecule expression and relatively high MGL expression are able to suppress TCR-mediated T cell activation through interaction with CD45 expressed by T cells, resulting in reduced cytokine production and proliferation. Conversely, LPS-matured DCs that initiate adaptive T cell immunity have no requirement for receptors with 'downregulatory' function, which explains why mature DCs are essentially MGL^{-}. The overall negative regulatory effect of MGL on T_{eff} cell activation along with its expression profile on tolerogenic APCs suggests that MGL controls unwanted T_{eff} cell activation in the steady state and during chronic inflammation.

All of the CD45 splice variants have similar phosphatase activity; yet the different isoforms have very restricted expression patterns[34]. How can changes in extracellular CD45 isoforms modulate CD45 function? One model proposes that the formation of homodimers inhibits phosphatase activity through interactions between an inhibitory 'structural wedge' and the catalytic site within the cytoplasmic CD45 phosphatase domains[35]. However, the crystal structure of CD45 seems incompatible with that wedge model[36]. Another straightforward explanation is receptor-mediated regulation of CD45 phosphatase activity. MGL specifically recognizes CD45 depending on the isoform and the cellular context in which an isoform is expressed. Thus, glycosylation patterns of single cells determine whether MGL is able to interact with and modulate CD45 function. Alternations in human CD45 splice variants are associated with autoimmune diseases, such as multiple sclerosis and systemic lupus erythromatosis[37,34]. It would be useful to determine whether these alterations lead to changes in glycosylation patterns, thus affecting MGL recognition and CD45 activity.

Although it was originally identified for its function in positively regulating antigen-receptor signaling through dephosphorylation of Src kinases, increasing evidence has indicated that CD45 can also negatively influence signaling pathways initiated by surface receptors[38]. Engagement of cell surface CD45 by monoclonal antibodies induces a programmed cell death pathway in lymphocytes[39-41]. Moreover, CD45 regulates apoptosis in peripheral T cells of transgenic mice expressing one single CD45 isoform[42]. Similarly, we detected CD45-dependent apoptosis in Jurkat cells after MGL-Fc binding; however cell death was equally dependent on CD3-triggering,

hinting at antigen-specific induction of cell death. Although we have demonstrated that MGL reduced primary T cell proliferation, we did not find substantial apoptosis in human T cells after triggering with MGL-Fc and anti-CD3, possibly because of the requirement for a 'pre-activation status' of the cells (data not shown). Some controversy exists regarding the requirement of CD45 phosphatase activity for eliciting cell death[39,40], and an apoptotic pathway acting through the production of reactive oxygen species has been proposed. The addition of reactive oxygen species scavengers could similarly block apoptosis induction in our system (data not shown). TCR-based signaling events can upregulate CD95 ligand expression, a process equally dependent on CD45[43]. Accordingly, our combined MGL-Fc–TCR stimulation might result in enhanced CD95-mediated programmed cell death, as nuclear condensation is observed in both apoptotic pathways[39].

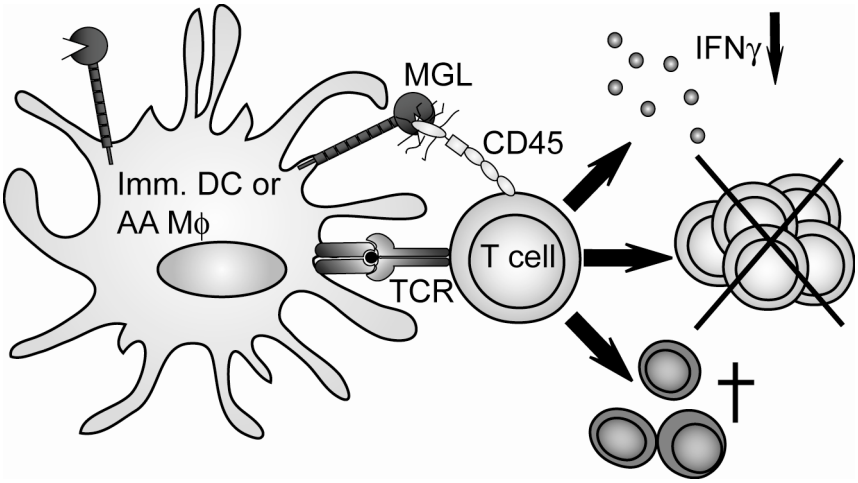

Figure 8. Model of MGL mediated control of effector T cell homeostasis. MGL binding to CD45 on T_{eff} cells negatively influences TCR-mediated signaling, leading to reduced proinflammatory cytokine production and proliferative responses, and even induction of T cell death.

The unique activating and suppressive features of CD45 make it a likely therapeutic target molecule, especially because antibodies to CD45RB potently inhibit T cell responses. In mice, CD45RB-bright $CD4^+$ T cell population include T_{eff} cells, some of which can induce autoimmunity or inflammatory bowel disease[44,45]. Treatment with monoclonal antibody to CD45RB prolongs long-term allograft survival and donor-specific tolerance in several murine renal and islet transplantation models[46]. A human antibody specific for CD45RO and CD45RB effectively inhibits antigen-specific and polyclonal T cell responses, mediated apoptosis of $CD4^+$CD45RO-bright RB-bright T cells and induced type 1 regulatory T cell development *in vitro*[47]. Such IL-10–producing regulatory T cells can likewise be generated by the differentiation of naive T cells in the presence of dexamethasone-treated APC[48].

The effects of anti-CD45RB therapy are very similar to the functional consequences of the MGL-CD45 interaction for T_{eff} cells. As CD45RB expression is retained on T_{eff} cells[17] and MGL selectively recognizes all CD45RB-containing isoforms, MGL might

be the natural receptor on APCs capable of mediating such processes. For further investigation of MGL-mediated immune suppression, MGL knockout mice would provide useful model systems. However, mice have two functional copies of the gene encoding MGL with carbohydrate recognition profiles distinct from that of human MGL[14], suggesting that mouse and human MGL have different ligands and possibly different immunological functions[49].

Our results have indicated a model of MGL-mediated control of T_{eff} cell function and have demonstrated a unique and previously unknown function for CLRs on APCs in the regulation of T cell homeostasis (Fig. 8). Compared with anti-CD45 antibodies, MGL-Fc showed an enhanced specificity, targeting only T_{eff} cells with properly glycosylated CD45. In contrast, anti-CD45RB antibodies bound and potentially affected all leukocytes expressing the B isoform, independently of cellular glycosylation patterns. Future investigations should evaluate the potential effectiveness of MGL in combating unwanted inflammatory responses in transplantation settings and autoimmune syndromes.

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CHAPTER 5

THE C-TYPE LECTIN MGL IMPEDES MIGRATION OF IMMATURE DENDRITIC CELLS BY MEDIATING RETENTION IN SKIN AND LYMPHOID ORGANS

Sandra J. van Vliet*, Lutz C. Paessens*, Venice C.M. Broks-van den Berg, Teunis B.H. Geijtenbeek and Yvette van Kooyk

*These authors contributed equally to this work

ABSTRACT

Dendritic cells are the most potent antigen presenting cells of the immune system that seed the peripheral tissues as precursors from the blood. In an immature state, dendritic cells sample their surroundings for incoming pathogens. Upon antigen encounter dendritic cells mature and migrate from the periphery to the T cell areas of the draining lymph node to induce adaptive immune responses. Migration of dendritic cells from the skin to the draining lymph node is regulated by the expression of chemokines and cell surface adhesion receptors. Here we demonstrate that the C-type lectin MGL, expressed by immature dendritic cells present in skin, interacts in an α-GalNAc-dependent manner with connective tissue surrounding small blood vessels. MGL binding does not induce migration; instead MGL$^+$ immature dendritic cells are retained through these α-GalNAc restraints. We also observed that sinusoidal and lymphatic endothelium of lymph node and thymus express α-GalNAc structures that allow MGL positive cells to remain at that site. In conclusion, in contrast to receptors that drive dendritic cell migration, MGL-α-GalNAc interactions facilitate retention and prevent the egress of dendritic cells out of the tissue.

INTRODUCTION

The correct positioning of immune cells is crucial for the orchestration of an effective immune response. This involves not only the regulation of cellular localization at strategic sites in the body, but also the control of cell mobility, allowing appropriate interactions to occur at the right time and the right place. From the blood, precursor dendritic cells (DCs) seed all peripheral tissues, where they, in an immature state, act as sentinels of the immune system by sampling their microenvironment for incoming microbes or changes in tissue homeostasis[1,2]. Upon pathogen encounter or stimulation by locally produced proinflammatory cytokines DCs undergo a maturational process and embark via the afferent lymphatics to the draining lymph node (LN)[3,4]. However, before they can enter the lymph vessels, DCs have to be released from their local constraints and retention signals. The upregulation of specific chemokine receptors, such as CCR7, licenses the DCs to respond to chemokine gradients that guide them to the LN sinuses, the entry site for DCs in the LN[3]. DCs have then to advance into the paracortex and engage naive T cells for subsequent T cell activation and differentiation. Therefore, the trafficking of DCs to and within the secondary lymphoid organs is a critical event in the induction of T cell immunity.

Different protein families, including chemokines, chemokine receptors and integrins, play crucial roles in regulating immune cell mobility. Functional defects in any of these proteins can impair the migratory capacities of DCs or lymphocytes and cause varied levels of immune dysregulation[5-7]. In addition, members of the C-type lectin family can participate in the recruitment of immune cells into tissues or lymphoid

organs. The important role for C-type lectins in leukocyte migration is exemplified by the leukocyte adhesion deficiency II (also known as congenital disorder of glycosylation IIc), in which the selectin ligand, the sialyl-Lewis X structure, is critically absent from leukocytes. The lack of sialyl-Lewis X leads to impaired tethering and rolling of immune cells on endothelial expressed selectins, thus resulting in a severe immune deficiency[8]. Selectins mediate lymphocyte interactions with the lymphatic endothelium as well, through the binding of L-selectin to the C-type lectin mannose receptor (MR)[9]. Moreover MR, Endo180 and langerin all bind components of the extracellular matrix, indicating that these interactions may contribute to trafficking or retention of DCs or macrophages in tissues[10-12]. The C-type lectin DC-SIGN has been described to facilitate DC migration and homing of DC precursors to the peripheral tissues through an interaction with endothelial ICAM-2[13], that expresses the Lewis Y carbohydrate structure (J.J. Gárcia-Vallejo, *manuscript submitted*).

Several lines of evidence suggest that the C-type lectin macrophage galactose-type lectin (MGL) could likewise be involved in the trafficking of macrophages and DCs that express this α/β-GalNAc-specific lectin[14]. In mice, mMGL expression is upregulated on migrating Langerhans cells (LCs) during lymphatic transit[15]. Furthermore, antibodies to mMGL can dampen the sensitization phase of contact hypersensitivity, suggesting a role for mMGL$^+$ in the migration of dermal DCs to the draining LN[16,17]. In humans, MGL is highly expressed on tolerogenic DCs, alternatively activated macrophages and on a CD1a$^+$ dermal DC subset that exhibits a chemotactic response towards CCR7 ligands[18-20].

Here, we demonstrate that MGL ligands are present on the sinusoidal and lymphatic endothelium of LN and thymus and on the connective tissue surrounding small blood vessels in the skin. MGL binding strongly correlated with the expression of the preferred MGL ligand, α-GalNAc-containing glycan structures, as visualized by staining with the α-GalNAc-specific snail lectin *Helix pomatia* agglutinin (HPA). Strikingly, instead of inducing migration, MGL mediated retention of human immature DCs, as blockade of MGL interactions enhanced DC trafficking and migration. Thus, MGL$^+$ DCs that reside in tissues are hampered in their chemotactic responses and only upon maturation, when MGL expression is downregulated, DCs will be released from these MGL-mediated restraints.

MATERIALS AND METHODS

Cells and reagents

Immature monocyte-derived DCs were cultured for 4-7 days from monocytes obtained from buffy coats of healthy donors (Sanquin, Amsterdam) in the presence of IL-4 and GM-CSF (500 U/ml and 800 U/ml respectively, Biosource, Camarillo, CA). HMEC-1 cells were cultured in MCDB 131 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 10 ng/ml epidermal growth factor and 1 μg/ml hydrocortisone. Human umbilical vein endothelial cells (HUVEC) were isolated as

previously described[21] and cultured in M199 medium (Cambrex, East Ruhterford, NJ) supplemented with 10% human serum, 10% newborn calf serum, 5 U/ml heparin and 5 ng/ml basic fibroblast growth factor. Polyacrylamide (PAA)-coupled glycoconjugates were purchased from Lectinity (Lappeenranta, Finland). Biotinylated *Helix pomatia* agglutinin (HPA) was purchased form Sigma Aldrich (St. Louis, Mo). Biotinylated *Maackia amurensis* agglutinin (MAA), *Sambucus nigra* agglutinin (SNA) and *Ulex europaeus* agglutinin-1 (UEA-1) were purchased from Vector Laboratories (Burlingame, CA). MGL-Fc, containing the human IgG1 Fc domain, was generated as previously described[14]. An MGL-murine Fc (MGL-mFc) fusion protein was generated by cloning the extracellular part of MGL (amino acids 61-289) into an pcDNA3 expression vector containing exon 1-3 of murine IgG2a-Fc[22]. MGL-mFc concentrations were determined by ELISA.

ELISA-based MGL-Fc binding assays

PAA-glycoconjugates were coated at 5 μg/ml on NUNC maxisorb plates (Roskilde, Denmark) overnight at room temperature. Plates were blocked with 1% BSA and MGL-mFc was added (0.5 μg/ml) for 2 hours at room temperature in the presence or absence of 10 mM EGTA. Binding was detected using a peroxidase-labeled anti-mouse IgG Fc antibody (Jackson, West grove, PA).

Immunohistochemistry

Cryosections of healthy tissues (7 μm) were fixed with 2% paraformaldehyde. MGL-mFc (25 μg/ml) or anti-MGL (18E4, 10 μg/ml) were added in TSM buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) and incubated for 2 hours at 37°C. Binding was detected with a secondary Alexa 594-conjugated goat anti-mouse IgG2a-specific antibody (Molecular Probes, Eugene, OR). Where indicated, sections were co-stained using primary mouse antibodies to Lyve-1[23,24] (lymphatic/sinusoidal endothelium) or antibodies to von Willebrand factor (vWF, vascular endothelium, Dako, Glostrup, Denmark) for 2 hours at 37°C, followed by a secondary Alexa 488-conjugated goat anti-mouse IgG1-specific antibody (Molecular Probes). Sections were counterstained using Hoechst.

Flow cytometry and MGL-Fc binding

Cells were incubated with primary antibody (5 μg/ml), followed by staining with a secondary FITC-labeled goat anti-mouse antibody (Zymed, San Francisco, CA) and analyzed on FACScalibur (BD Biosciences, San Diego, CA). To assess the expression of carbohydrate epitopes on the cell surface, cells were incubated in 10 μg/ml of the biotinylated plant/snail lectins in TSM supplemented with 0.5% BSA for 30 min at 37°C, followed by staining with a Alexa 488-conjugated streptavidin (Molecular Probes) and analyzed on FACScalibur. To analyze MGL ligand expression, cells were incubated with MGL-Fc (10 μg/ml) in TSM supplemented with 0.5% BSA for 30 min at 37°C, followed by staining with a secondary FITC-labeled anti-human Fc antibody (Jackson, West grove, PA) and analyzed on FACScalibur. In blocking experiments,

MGL-Fc was preincubated for 15 minutes at room temperature with 10 mM EGTA, 100 mM free GalNAc (Sigma Aldrich, St. Louis, MO), 20 μg/ml of the lectins or 20 μg/ml anti-MGL antibodies.

Migration assays

Transwell 24-well plates (8 μm pore, Greiner Bio-one, Frickenhausen, Germany) were coated with 1% gelatine for 1 hour at 37°C. HMEC-1 cells (70 x 10^3) were seeded on the inserts and after 24 hours 200 x 10^3 DCs were added to the monolayer of endothelial HMEC-1 cells. The lower chamber contained human RANTES (100 ng/ml, Biosource). After 2 hours at 37°C, the number of transmigrated DCs (lower chamber) was determined by flow cytometry. Transendothelial migration was measured in the presence or absence of blocking or isotype-matched antibodies (20 μg/ml). DC migration on coated PAA-glycoconjugates (5 μg/ml) was studied using time-lapse video microscopy. 45 x 10^3 DCs were added to the plates, allowed to settle for 30 minutes and the number of migrating DCs was assessed for a 30 minute period. The migration assay was conducted in the presence or absence of blocking antibodies (20 μg/ml). Migration was scored as the percentage of cells displaying spatial movement on the coating, accompanied with changes in cell shape.

RESULTS

Detection of MGL ligands in healthy human tissue

In order to explore the distribution of MGL ligands in tissue we generated a recombinant protein consisting of the extracellular domain of MGL fused to the mouse IgG2a Fc tail. To confirm that the recombinant MGL-mFc has similar binding properties as cellular MGL[14], MGL-mFc binding assays were performed to coated PAA-glycoconjugates. MGL-mFc recognized both α-GalNAc and the LacdiNAc epitope (LDN or GalNAcβ1-4GlcNAc; Fig. 1). MGL-mediated binding was completely inhibited in the presence of the Ca^{2+}-chelator EGTA, showing the involvement of the MGL carbohydrate recognition domain. MGL-mFc did not interact with Lewis X or mannose. Thus, MGL-mFc displays an identical carbohydrate recognition profile as MGL expressed by human immature DCs[14].

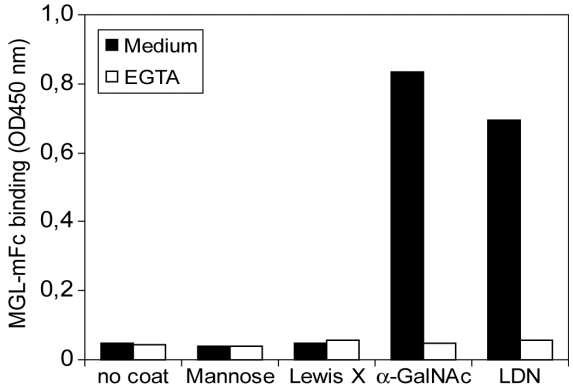

Figure 1. MGL-mFc recognizes α-GalNAc and LDN structures. MGL-mFc binding was determined by ELISA. Standard deviation <0.05 OD 450 nm. One representative experiment out of three is shown.

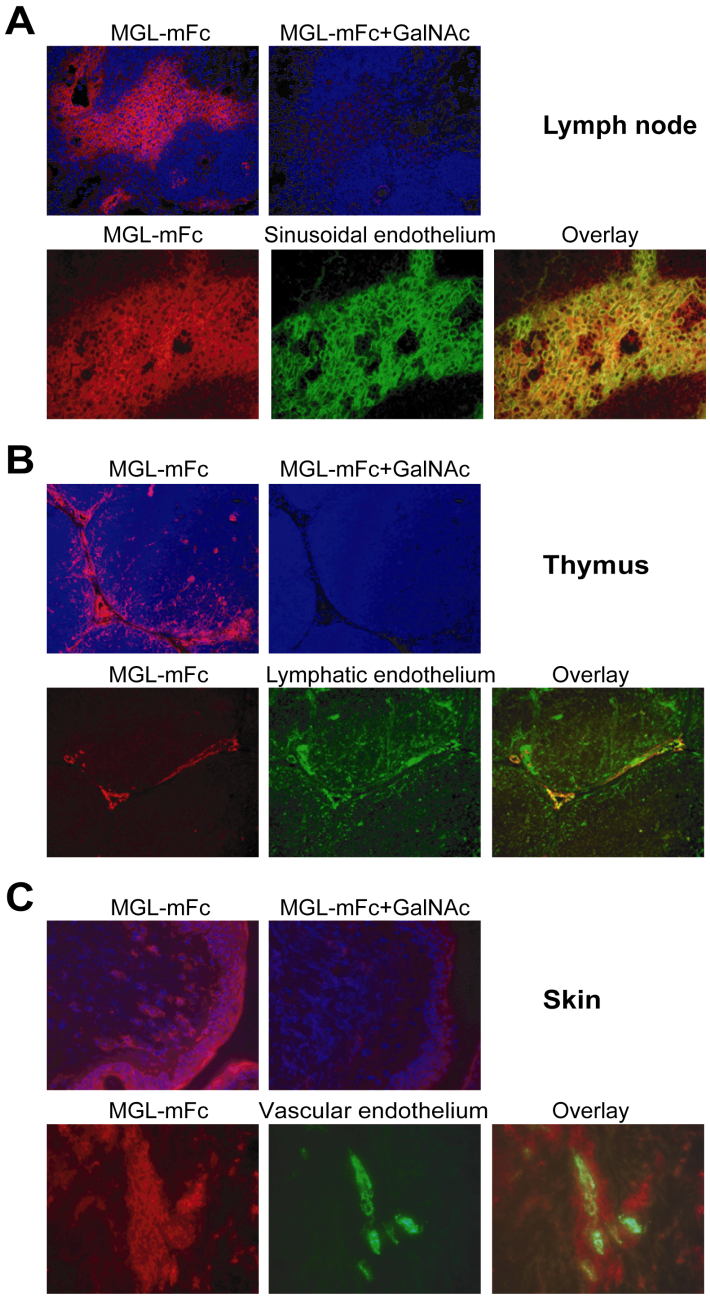

Figure 2. MGL ligands can be detected in human tissues. MGL-mFc (red) specifically interacted with the sinusoidal endothelium (LYVE-1, green) of LN (A) and with the lymphatic endothelium (LYVE-1, green) of the thymus (B). In skin (C) the connective tissue surrounding blood vessels (vWF, green) were stained by MGL-mFc (red). No staining was observed in the presence of free GalNAc mono-saccharides, showing the specificity of the MGL binding. Nuclei are visualized in blue. One out of two donors is shown. Original magnifications 200x and 400x.

We labeled cryosections of healthy human tissues with MGL-mFc to investigate the expression of potential MGL ligands *in situ* (Fig. 2). In LN and thymus MGL ligands could be detected on the sinusoidal and lymphatic endothelium, respectively (Fig. 2A and B). In contrast, in skin lymphatic vessels were not stained by MGL-mFc (data not shown). In skin, MGL interacted with the connective tissue surrounding small blood vessels in the dermis (Fig. 2C). In addition to the tissues shown in figure 2, MGL-mFc labeled all epithelial cells and mucus layer of the small intestine, endothelial cells of the central arteriole in the liver and an unidentified subpopulation of cells in the splenic red pulp (data not shown). In all tissues MGL-mFc staining was completely abolished by the addition of free GalNac monosaccharides, demonstrating the dependence on the MGL carbohydrate

recognition domain for binding (Fig. 2).
Next, we assessed whether MGL$^+$ antigen presenting cells (APCs) could be detected in the same tissues that harbour the MGL ligands. In LN MGL$^+$ APCs are located just below the lymph node sinusoidal endothelium (Fig. 3). In thymus MGL$^+$ APCs are found in the interlobular space, where also the lymphatic vessels are situated. In addition, MGL$^+$ APCs can be observed throughout the dermis.
In conclusion, the MGL protein and its ligands are expressed in close proximity at similar anatomical locations. The specific MGL binding to vascular structures prompted us to further investigate the role of MGL in the migration process of immature DCs.

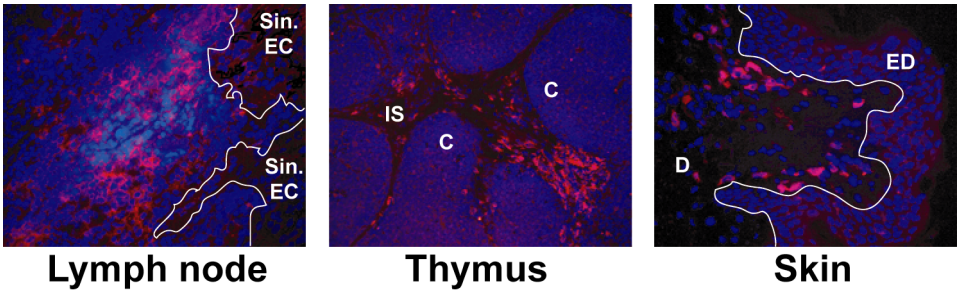

Figure 3. MGL expression in lymph node, thymus and skin. Human tissue sections of lymph node, thymus and skin were stained with anti-MGL (red). Nuclei are visualized in blue. Sin. EC, sinusoidal endothelial cells; C, cortex; IS, interlobular space; EP, epidermis; D, dermis. One out of two donors is shown. Original magnification 400x.

MGL specifically interacts with endothelial cells in an α-GalNAc-dependent manner
To investigate a potential role for MGL in DC migration, we examined binding of MGL to cultured endothelial cells. Human umbilical vein endothelial cells (HUVECs), a model system for endothelial cells lining blood vessels, were not bound by MGL-Fc (Fig. 4A). TNFα, IL-4 or IFNγ stimulation of HUVECs did not upregulate any MGL ligand expression (data not shown). These results are in agreement with the data presented in figure 2, in which we did not observe any MGL-mFc binding to the endothelial cells lining the small blood vessels of the skin. In contrast, HMEC-1 cells, a human endothelial cell line that expresses several lymphatic endothelial markers[25], strongly interacted with MGL-Fc (Fig. 4B). MGL binding could be blocked by the addition of the Ca^{2+}-chelator EGTA, free GalNAc monosaccharides or anti-MGL antibodies, demonstrating the specificity of this interaction.
To explore the nature of the MGL ligand on these cells, we investigated by flow cytometry, using well-characterized plant/invertebrate lectins, which glycan epitopes are present on HMEC-1 cells. Previous studies have demonstrated a strong correlation between MGL binding and the expression of glycan epitopes recognized by the α-GalNAc-specific roman snail lectin *Helix pomatia* agglutinin (HPA)[14]. Although the carbohydrate recognition patterns of MGL and HPA show a high

degree of similarity, HPA recognized some additional terminal GlcNAc-containing glycans[26]. Indeed, HMEC-1 expressed HPA-reactive glycan structures (Fig. 4C). HPA binding could be blocked by the addition of free GalNAc, indicating that HPA recognized α-GalNAc containing glycans on the HMEC-1 cells. In addition α1-2 linked fucose, α2-3 and α2-6 linked sialic acid structures are present on HMEC-1 cells, as visualized by the reactivity of *Ulex europaeus* agglutinin-1 (UEA-1), *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) respectively (Fig. 4C). However, when these lectins were used to block MGL-Fc binding, only HPA could significantly interfere with the MGL-HMEC-1 interaction, suggesting that an α-GalNAc-containing glycoprotein or –lipid constitutes as the ligand for MGL on HMEC-1 cells (Fig. 4D). Since HPA could only inhibit MGL binding for 50%, additional MGL binding determinants on HMEC-1 cells exist that are probably composed of terminal β–GalNAc structures[14].

Next, we explored whether the distribution of MGL ligands in tissue also correlated with HPA reactivity. Since HPA can block MGL binding (Fig. 4D), we performed a double labeling on tissues by employing HPA together with the specific endothelial

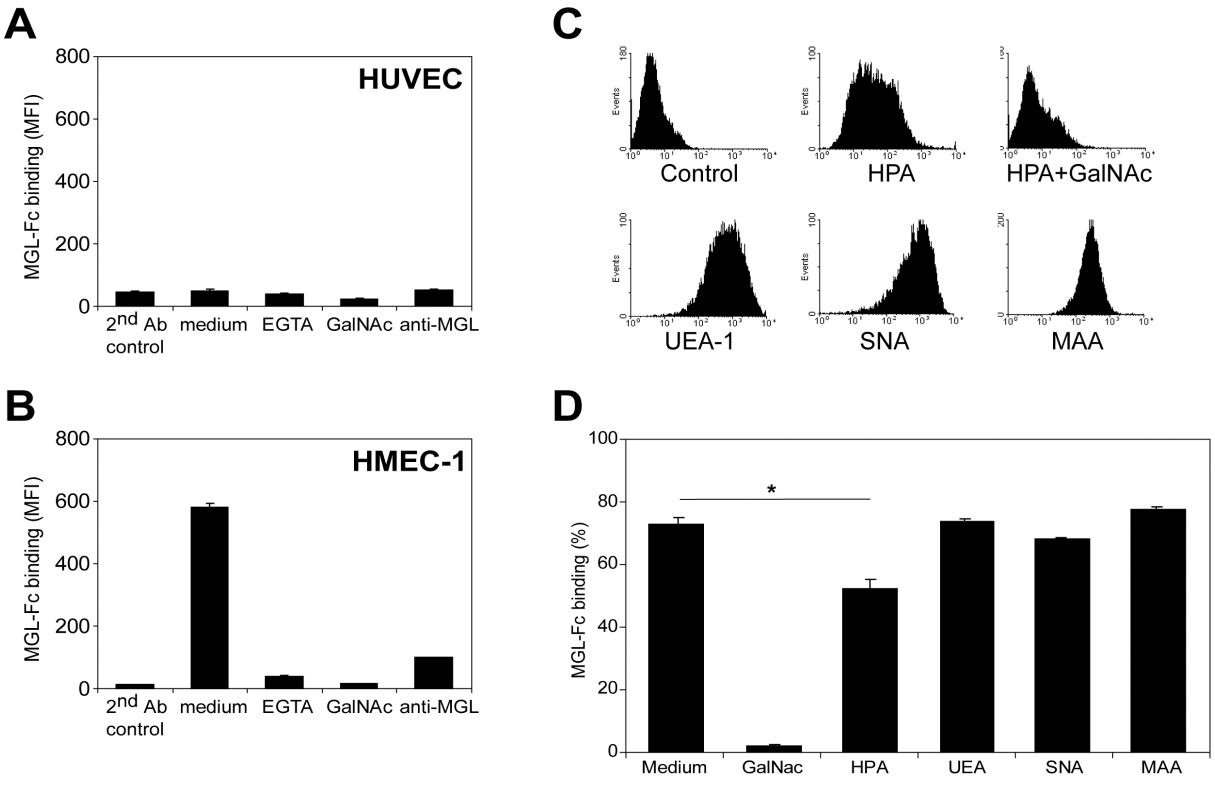

Figure 4. MGL specifically recognizes HMEC-1 endothelial cells. Binding of MGL-Fc to primary HUVEC (A) or HMEC-1 (B) was determined by flow cytometry. MGL-Fc binding to HMEC-1 can be blocked by EGTA, free GalNAc monosaccharides and anti-MGL antibodies. (C) HMEC-1 cells express α-GalNAc, α1-2 linked fucose, α2-6 linked sialic acid and α2-3 linked sialic acid, as stained by the lectins HPA, UEA-1, SNA and MAA respectively. (D) MGL-Fc binding can be partially blocked by the α-GalNAc–specific lectin HPA. * indicates a significant difference between the MGL-Fc binding and the MGL-Fc binding in the presence of HPA ($p<0.05$). All results are representative for three independent experiments.

cell markers. Strikingly, although HPA stained additional vessels in LN and thymus, it displayed a complete overlap with the sinusoidal and lymphatic endothelium (Fig. 5A and B). These subpopulations of endothelial cells were also recognized by MGL (Fig. 2A and B). Dermal lymphatic vessels were not positive for HPA (data not shown), nor were they recognized by MGL (Fig. 2C). In contrast, in skin HPA appeared to label the connective tissue or extracellular matrix, with higher intensities observed just below the epidermis and surrounding the blood vessels (Fig. 5C). The latter structures also bound MGL-mFc (Fig. 2C). The GalNAc block of MGL binding to tissues (Fig. 2) combined with the specific HPA staining (Fig. 5) strongly indicate that MGL interacts with endothelial cells in LN or thymus and the connective tissue in skin in an α-GalNAc-dependent manner.

MGL mediates retention of immature dendritic cells

To evaluate a possible involvement of MGL in DC migration, immature DCs were cultured from monocytes. Immature DCs expressed moderate levels of MGL (Fig. 6A). We measured the capacity of these DCs to transmigrate across an HMEC-1 monolayer. In response to the chemokine RANTES, transmigration of immature DCs was enhanced about 3-fold and largely dependent on β2-integrins, as shown by the block using anti-β2 antibodies (Fig. 6B)[27]. Strikingly, anti-MGL significantly increased transmigration compared to isotype control antibodies (Fig. 6B).

To study if α-GalNAc is sufficient to support DC migration, we followed DC mobility on glycoconjugate-coated plates with time-lapse videomicroscopy. Migration was scored as the percentage of cells displaying spatial movement on the coating, accompanied with changes in cell shape. DCs did not interact with the glucitol or galactose coating (Fig. 6C). In contrast, DCs firmly adhered to Lewis X and Man3, which inhibited DC mobility. Surprisingly, on GalNAc, DCs displayed a strong migratory movement, while continuously interacting with the coated surface (Fig. 5C). Similar to the transmigration experiments, anti-MGL antibodies significantly increased the percentage of migrating DCs on the GalNAc-coating, as well as the speed by which the DCs moved (Fig. 6D and data not shown). The increased migration was not due to a nonspecific effect on the immature DCs, as isotype control antibodies did not induce enhanced mobility. Furthermore, the anti-MGL antibodies did not have any enhancing effect on DC migration on Lewis X (Fig. 6D). DCs incubated with anti-MGL antibodies still interacted with the GalNAc-coated surface, suggesting that immature DCs express another GalNAc-specific receptor in addition to MGL.

Based on our *in vitro* migration experiments, it is likely that expression of MGL on immature DCs and the presence of GalNAc sugars hamper DC migration and favors DC retention in the tissue. Blocking MGL function or downregulation of its expression due to DC maturation[20], can relieve DCs from the GalNAc constraints and enhance the migratory capacities of DCs.

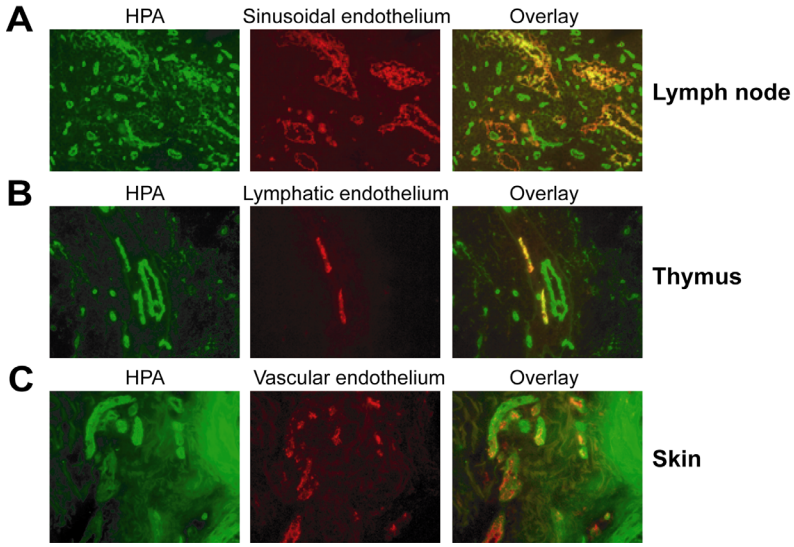

Figure 5. Endothelial structures express GalNAc epitopes as visualized by HPA staining. HPA (green) specifically interacted with the sinusoidal endothelium (LYVE-1, red) of LN (A) and lymphatic endothelium (LYVE-1, red) in thymus (B), whereas in skin (C) the connective tissue surrounding blood vessels (vWF, red) were stained by HPA (green). Original magnification 400x.

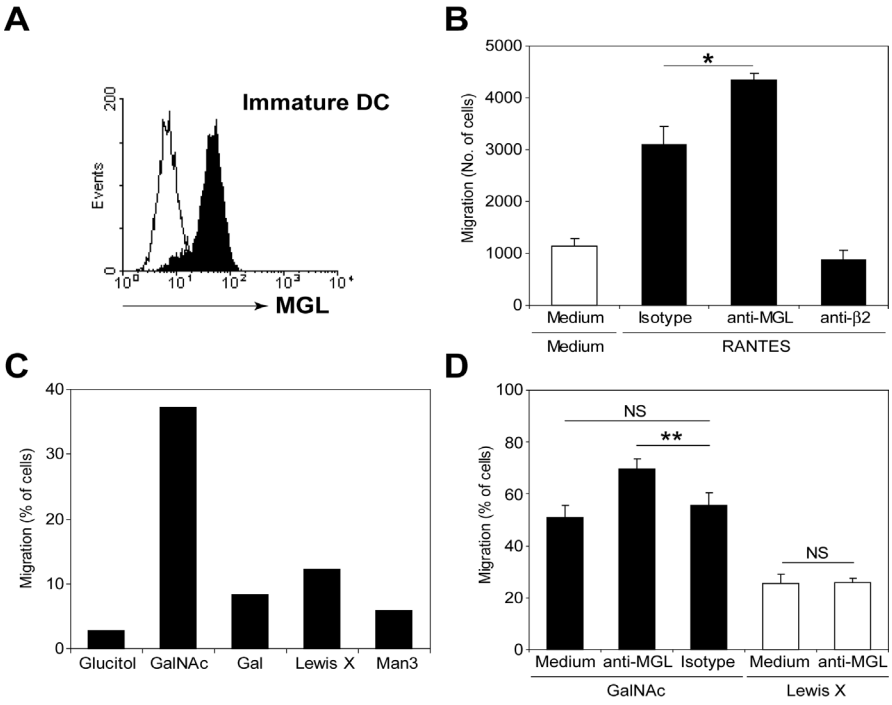

Figure 6. MGL mediates retention of immature DCs. (A) MGL expression on immature DCs was determined by flow cytometry. Open histogram, isotype control; filled histogram MGL staining (B) Transendothelial migration of immature DCs across an HMEC-1 monolayer in response to a RANTES gradient in the presence of antibodies to MGL, β2-integrin or isotype control antibody. * indicates a significant difference between the isotype control and the anti-MGL ($p<0.05$). (C and D) DC migration on glycoconjugate-coated plates was measured using time-lapse video microscopy. Anti-MGL antibodies increase DC migration on GalNAc, whereas they have no effect on the Lewis X coating. ** indicates a significant difference between isotype control and the anti-MGL antibodies ($p<0.01$). NS, not significant. All results are representative for two independent experiments.

DISCUSSION

In recent years our understanding on DC trafficking has greatly increased, yet little is known about which mechanisms actively retain immature DCs in the tissue[3,4]. Here we demonstrate that the C-type lectin MGL facilitates α-GalNAc–dependent retention of immature DCs. Since blocking MGL function enhanced the migratory capacities of DCs, it is tempting to speculate that MGL-GalNAc interactions maintain the localization of DCs at strategic sites and prevent DC egress.

In the thymus and lymph node MGL specifically recognized the lymphatic and sinusoidal endothelium respectively, indicating that *in situ* those endothelial cells express GalNAc structures that serve as MGL binding determinants. Recently, Bonasio *et al* showed that circulating DCs home to the thymus via the blood and there contribute to the induction of central tolerance[28]. Moreover, a small percentage of peripheral DCs can re-enter circulation through efferent lymphatics[29]. Thus, MGL binding to lymphatic vessels in the thymus could impair DC exit from the thymus, allowing these immature DCs to interact with developing thymocytes. Lymph node sinuses have been proposed to function as molecular sieves for filtering lymph-borne antigens[24]. The close proximity of MGL$^+$ cells to the sinusoidal endothelial cells suggest that these MGL$^+$ APCs are positioned at that site for capturing antigens. The MGL-sinus interaction would then facilitate such strategic localization. In skin MGL interacted with the connective tissue surrounding the small blood vessels. Precursor DCs enter the skin via the bloodstream. Binding of these extracellular matrix components might avert DCs from transmigrating back into blood. Further studies will be needed to identify the endothelial glycoproteins or –lipids that carry the GalNAc determinants for MGL binding and to determine how these retention molecules are regulated.

Our data seems to contradict previous reports investigating the migratory capacities of mouse mMGL$^+$ DCs. Anti-mMGL antibodies blocked the migration of mMGL$^+$ dermal cells from skin explants, strongly indicating that at least in mice mMGL can support migration[17]. Since mMGL possesses a broader carbohydrate recognition profile compared to human MGL[14,30], mMGL might possess additional ligands as well that facilitate the migratory capacities of mMGL. Migrating mouse LCs upregulate mMGL expression during lymphatic transit, suggesting a role for mMGL in lymph vessel migration[15]. However, the interaction between mMGL and the lymphatics could also slow down LCs, as we have observed for human immature DCs, allowing full maturation to occur before these cells reach the skin-draining LN. In accordance, mature LCs lose cell surface expression of mMGL[15].

The classical paradigm states that in the periphery immature DCs support pathogen recognition and that priming of naive T cells occurs in the LN by mature DCs. MGL is only expressed by immature DCs. Via this MGL expression immature DCs may be actively retained in the peripheral tissue, where they are optimally situated for microbial patrol. Upon pathogen encounter and Toll-like receptor engagement these DCs will mature and subsequently lose MGL expression[19]. The loss of MGL

expression would release the maturing DCs from local GalNAc restraints and LN migration would be enhanced.
In contrast, MGL-mediated retention could also hamper the induction of appropriate immune responses. Several adenocarcinomas show aberrant glycosylation patterns, whereby short O-glycans, such as the Tn antigen (α-GalNAc-Ser/Thr), are highly upregulated[31]. This Tn antigen is one of the high affinity carbohydrate ligands for human MGL. Thus, the tumor microenvironment might prevent egress of MGL^+ APCs and indeed we observed large infiltrates of MGL^+ DCs within human colon carcinomas (data not shown). These MGL^+ DCs can then block the cytolytic function of incoming tumor-specific $CD8^+$ T cells[20].
Several research groups have employed DCs for vaccination immunotherapy of tumor patients[32,33]. Unfortunately, the migration of *ex vivo* generated DCs is rather inefficient[34], whereby most DCs remain trapped at the injection site. Especially, immature DCs migrated at extremely low frequencies, which might be partially attributed to the MGL expression on these cells. Although researchers now routinely use mature MGL^- DCs for active tumor immunity, DC immunotherapy could also be useful to induce peripheral tolerance. Such applications of immature DCs in for instance transplantation of autoimmune settings might likewise be impaired due to local retention of these immature cells via MGL-α-GalNAc intractions.

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CHAPTER 6

ESSENTIAL ROLE OF TYROSINE-5 IN MGL-MEDIATED INTERNALIZATION AND ANTIGEN PRESENTATION BY DENDRITIC CELLS

Sandra J. van Vliet, Corlien A. Aarnoudse, Venice C.M. Broks-van den Berg, Martine Boks, Teunis B.H. Geijtenbeek and Yvette van Kooyk

Submitted for publication

ABSTRACT

Professional antigen presenting cells are essential for the initiation of adaptive immune responses; however they also play a vital role in the maintenance of tolerance towards self-antigens. C-type lectins can function as antigen receptors, by capturing carbohydrate ligands for processing and presentation. Here, we focused on the dendritic cell (DC)-expressed macrophage galactose-type lectin (MGL), a C-type lectin with a unique specificity for terminal GalNAc residues, such as the tumor-associated Tn antigen. Soluble model antigens are efficiently internalized by MGL and subsequently presented to responder $CD4^+$ T cells. The tyrosine-5 residue in the YENF motif, present in the MGL cytoplasmic domain, was essential for the MGL-mediated endocytosis. In conclusion, MGL contributes to the antigen processing and presentation capacities of DCs and may provide a suitable target for the initiation of anti-tumor immune responses.

INTRODUCTION

Dendritic cells (DCs) efficiently capture antigens for processing and presentation to naive T cells[1]. In an immature state DCs line all peripheral tissues, where they continuously inspect their surroundings for incoming microbes or changes in local conditions. Upon pathogen encounter DCs undergo maturation, consisting of a short enhanced wave of antigen uptake, followed by the upregulation of chemokine receptors, MHC molecules and costimulatory molecules[2,3]. Mature DCs show enhanced migration towards afferent lymph nodes for initiation of naive T cell responses. However, even under steady state conditions semi-mature DCs continuously migrate to the lymph node to present self-antigens to T cells, thereby actively inducing T cell tolerance towards harmless self-antigens[4].

DCs express many different types of molecules that participate in the scavenging of antigens, such as Fc-receptors, scavenger receptors and C-type lectins. Several C-type lectins, which recognize carbohydrate ligands in a Ca^{2+}-dependent manner, have been demonstrated to function as efficient antigen receptors[5-7]. Specialized internalization motifs in the C-type lectin cytoplasmic domain facilitate endosomal/lysosomal targeting, although the pathways for entry vary among the different C-type lectins studied[5,8,9]. The mannose receptor (MR) utilizes a di-aromatic sequence (YF) for endosomal sorting, whereas DC-SIGN-mediated internalization is regulated by a dileucine motif (LL)[5,10]. Strikingly, the internalization process is equally influenced by the ligand. The intracellular trafficking of Dectin-1 depends on the size of the interacting β-glucan[8]. Although DC-SIGN carbohydrate ligands are effectively targeted and degraded in the lysosomal compartment after binding[11], some pathogens are partially able to block this pathway[12,13].

The uptake of exogenous ligands via C-type lectins, such as DC-SIGN, MR and Dectin-1, results in presentation of antigenic peptides in MHC class II and even cross-presentation in MHC class I[5,6,14,15]. The absence of overt immune activation after

C-type lectin targeting in the absence of concomitant danger signals, e.g. a Toll-like receptor stimulus[16,17], led to the hypothesis that C-type lectins are the predominant antigen receptors in the steady state, thereby contributing to maintaining homeostasis control[18]. However, also pathogens that specifically bind C-type lectins can be processed and presented, thereby promoting immunity instead of tolerance[19].
The human C-type lectin macrophage galactose-type lectin (MGL) is expressed by immature DCs and macrophages[20-22]. In skin MGL is a marker for the $CD1a^+$ dermal DCs, a cell type with enhanced ability to stimulate naive T cells compared to other dermal APC subsets[23]. Human MGL has an exclusively specificity for terminal GalNAc-residues such as found in glycoproteins of the helminth parasite *Schistosoma mansoni,* filoviruses and in the tumor-associated antigens[20,24]. Especially the Tn antigen or α-GalNAc-Ser/Thr moiety, which is expressed by 90% of all carcinomas, is bound by MGL with high affinity[20,25]. Furthermore, via the MGL-CD45 interaction, MGL functions as a negative regulator of effector T cells[26]. The presence of two putative internalization motifs in the MGL cytoplasmic tail, a classical tyrosine-based YENF motif and a partial dileucine motif[27], prompted us to investigate whether MGL might function in antigen capture and processing by DCs. Although MGL-mediated internalization has been demonstrated by Higashi *et al*[28], little is known about which motifs might facilitate MGL entry or whether antigens endocytosed by MGL are available for processing and presentation. Here we demonstrate that MGL mediates uptake of soluble ligands and that the cytoplasmic YENF motif is indispensable for MGL uptake. Furthermore, soluble antigens, which have been endocytosed by MGL, are efficiently presented to responder $CD4^+$ T cells.
In conclusion, DC-expressed MGL can participate in the generation of immune responses to antigens that are specifically endocytosed by this receptor. Future studies should address whether MGL might be a suitable target for selective delivery of antigens to DCs.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were used: anti-MGL (18E4, 1G6.6[21] and MLD-1, kindly provided by dr. T. Irimura[28]), anti-DC-SIGN (AZN-D1[42]) and anti-ICAM-2 (12A2, isotype control[5]). Biotinylated polyacrylamide (PAA)-coupled glycoconjugates were purchased from Lectinity (Lappeenranta, Finland).

Cells

Immature monocyte-derived DCs were cultured for 4-7 days from monocytes obtained from buffy coats of healthy donors (Sanquin, Amsterdam) in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml). The phenotype of the cultured DCs was confirmed by flow cytometric analysis. DC consistently expressed high levels of MHC class I and II and low levels of CD80 and CD86.
CHO and CHO-MGL cells were maintained in RPMI 1640 medium (Invitrogen,

Carlsbad, CA) containing 10% Fetal Calf Serum. Mutations in the cDNA encoding MGL were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and pRc/CMV-MGL plasmid according to the manufacturer's protocol. The cytoplasmic tail deletion mutant was generated by replacing the entire cytoplasmic region by a new protein initiation codon. Plasmid sequencing confirmed the introduction of the mutations in the MGL cytoplasmic region (Fig. 1). Stable CHO-MGL transfectants were generated using lipofectamin (Invitrogen) and selected by the addition of 2 mg/ml Geneticin (Invitrogen). MGL positive cells were sorted using the MoFlo (DAKOcytomation, Glostrup, Denmark).

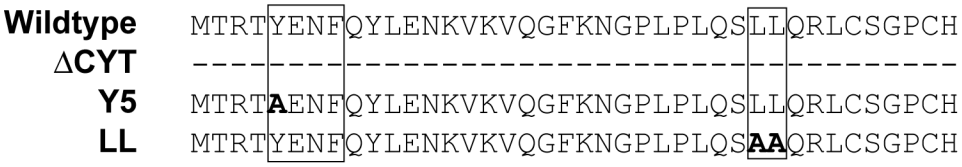

Figure 1. Cytoplasmic domain mutants of human MGL.

Flow cytometry, beads adhesion and internalization assays

Cells were incubated with primary antibody (5 μg/ml), followed by staining with a secondary FITC-labeled anti-mouse antibody (Zymed, San Francisco, CA) and analyzed on FACScalibur (BD Biosciences, San Diego, CA).

Streptavidin coated fluorescent beads (488/645 nm, Molecular Probes, Eugene, OR) were incubated with 1 μg of the PAA-coupled GalNAc. Fluorescent bead adhesion assay was performed as previously described[43] and analyzed on FACScalibur and presented as the percentage of cells which have bound the fluorescent beads.

Immature DCs were incubated with antibodies (10 μg/ml) or biotinylated PAA-glycoconjugates (5 μg/ml) in TSA (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.5% BSA) for 1 h on ice and subsequently washed. Cells were incubated at 37°C for various time points, placed on ice, and incubated with FITC-conjugated secondary antibodies or Alexa 488-labeled streptavidin (Molecular probes). To control for off rate of the antibodies at 37°C, cells were fixed before antibody binding to prevent membrane transport. Cells were analyzed using flow cytometry, and the relative differences in mean fluorescence intensities were compared.

MGL-Fc adhesion assay.

PAA-coupled GalNAc was coated (5 μg/ml) on NUNC maxisorb plates (Roskilde, Denmark) overnight at room temperature. Plates were blocked with 1% BSA and MGL-Fc was added (0.5 μg/ml in TSA) for 2 hours at room temperature in the presence or absence of 10 mM EGTA. Experiments were performed at the indicated pH. Binding was detected using a peroxidase-labeled anti-human IgG Fc antibody (Jackson, West grove, PA).

Antigen presentation

HD7, a $CD4^+$ T cell that recognizes a peptide derived from mouse IgG1 antibodies in HLA-DR0101/DQw1, was used[44]. Immature DCs (20 x 10^3/well) from a typed donor were preincubated with serial dilutions of antibodies and cocultured with 80 x 10^3 T cells. After 48 hours, IFNγ production by the T cells was measured by ELISA according to the manufacturer's protocol (Biosource international,.Camarillo, CA).

RESULTS

MGL mediates rapid internalization of ligands

The presence of two putative internalization motifs (YENF and LL) in the cytoplasmic domain of human MGL prompted us to investigate whether MGL expressed on DCs functions in antigen internalization and presentation. The C-type lectin DC-SIGN, known to rapidly internalize after ligand binding[5], was used as a positive control for endocytic uptake by DCs. Human monocyte-derived DCs expressed moderate levels of MGL and high levels of DC-SIGN (Fig. 2A). To investigate MGL-mediated internalization, we employed antibodies directed against the MGL carbohydrate recognition domain that mimic ligand binding. Antibodies to MGL were internalized from the DC surface at a similar rate as anti-DC-SIGN antibodies (Fig. 2B). The observed entry was not the result of an enhanced antibody off-rate at 37°C, since no decrease in antibody staining was seen on fixed DCs. Next, we determined whether soluble carbohydrate ligands are endocytosed by MGL as well. The MGL ligand PAA-GalNAc[20] and the DC-SIGN ligands PAA-Lewis X and PAA-Mannose[11] were internalized with faster kinetics from the cell surface compared to antibodies (Fig. 2C). These data demonstrate that, similar to DC-SIGN, antigens bound by MGL are rapidly endocytosed from the DC surface.

The YENF motif is essential for MGL-mediated endocytosis

To identify motifs involved in the MGL-mediated internalization process, we generated a complete cytoplasmic deletion mutant and MGL cytoplasmic domain mutants by site-directed mutagenesis (Fig. 1). Stable cell lines were generated by transfecting the constructs encoding wildtype and mutant MGL into CHO cells. MGL mutants were expressed at the cell surface at comparable levels as wildtype MGL (Fig. 3A).

Furthermore, mutating or deleting the cytoplasmic tail did not affect ligand recognition, as all MGL-transfectants displayed equal binding to GalNAc-coated beads (Fig. 3B), demonstrating that the cytoplasmic region is not involved in ligand binding. MGL binding could be inhibited by addition of the Ca^{2+}-chelator EGTA, confirming the specificity of this interaction.

Antibodies to MGL are internalized with similar kinetics on immature DCs and CHO-MGL (compare fig. 2B and fig. 3C). As expected, deletion of the entire MGL cytoplasmic tail (MGL ΔCYT) abrogated MGL-mediated endocytosis (Fig. 3C). Disruption of the YENF motif (MGL Y5) completely blocked internalization, whereas

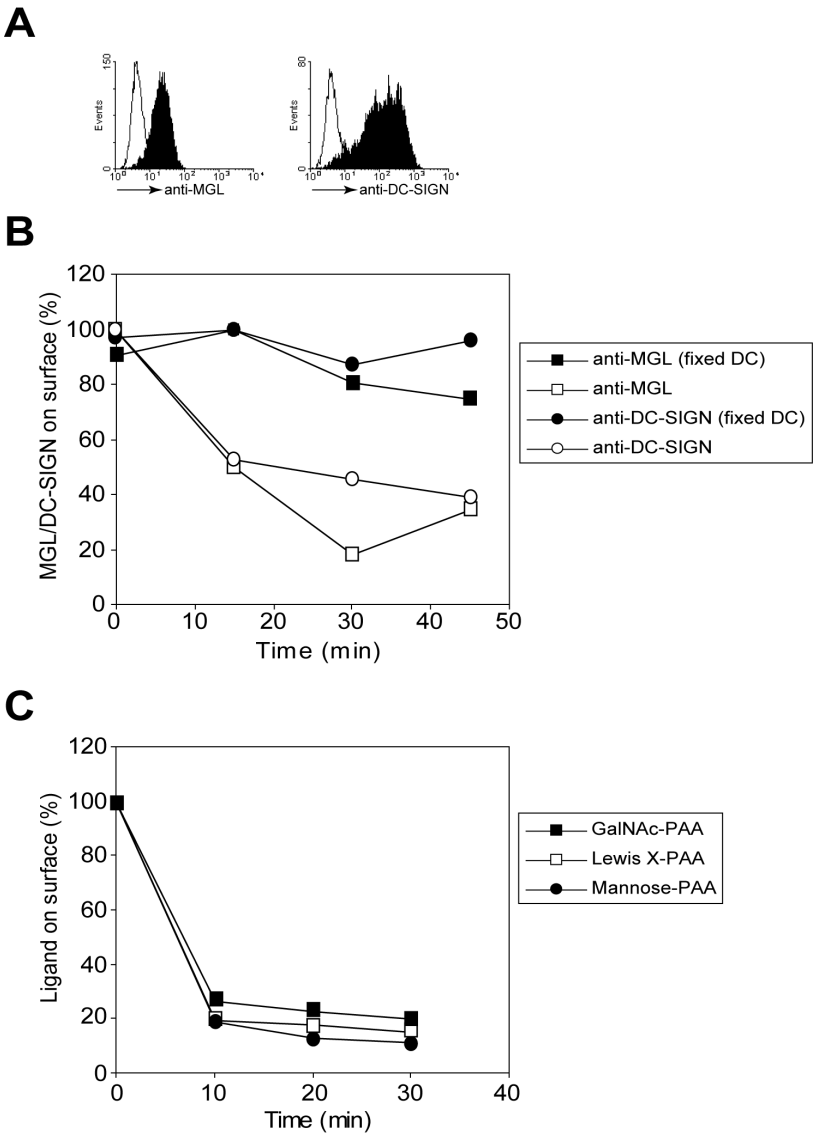

Figure 2. Ligand binding induces endocytosis of MGL. (A) Expression of MGL and DC-SIGN on human monocyte-derived DCs as measured by flow cytometry. (B) Internalization of cell surface bound antibodies to MGL and DC-SIGN as measured by flow cytometry on immature DCs. Fixed cells were included to control for the antibody off-rate at 37°C. Values represent mean of duplicates. (C) Internalization of cell surface bound ligands to MGL (GalNAc-PAA) and DC-SIGN (PAA-Mannose and PAA-Lewis X) as measured by flow cytometry on immature DCs. Maximal binding was set to 100% and corresponds to 30% of the cells binding to PAA-GalNAc and 98% binding of PAA-Lewis X and PAA-mannose. Values represent mean of duplicates. Results are representative for three independent experiments.

mutating the dileucine motif (MGL LL) reduced internalization by 45%. Thus, the YENF motif directly facilitates and is therefore essential for MGL-mediated internalization.

MGL captures antigens for processing and presentation

During transit through the endosomal/lysosomal pathway lectins and their cargo are exposed to a lowering pH gradient. An important property of recycling C-type lectin receptors, such as the MR, is their ability to dissociate from their cargo at low pH, enabling recycling of the receptor to the cell surface. We analyzed MGL binding to its ligand GalNAc at different pH to investigate at which pH MGL dissociates from its antigens. The pH used mimics the pH of the different intracellular compartments. The MGL-Fc-GalNAc interaction was disrupted at pH values <6.0, indicating that MGL can dissociate from its ligand in the late endosomal compartments and potentially recycle back to the cell surface (Fig. 4A). Lectins that continuously recycle, such as the MR, often possess a large intracellular pool[29]. Also the MGL protein is

mainly located in intracellular vesicles (data not shown)[30]. However, so far we have not been able to demonstrate any MGL recycling (data not shown). Whereas MGL lost all ligand binding at pH 5.5 (Fig. 4A), related C-type lectins DC-SIGN and MR still display some residual binding at pH 5.5 and only completely dissociate from their ligands at pH 5[5,31].

To investigate whether MGL-mediated endocytosis delivers antigens to MHC molecules for presentation to T cells, we employed a $CD4^+$ T cell clone that recognizes a peptide derived from mouse IgG1. Immature DCs from a donor with a compatible haplotype were preincubated with serial dilutions of MGL antibodies and their capacity to activate T cells was investigated. Incubation of DCs with an IgG1

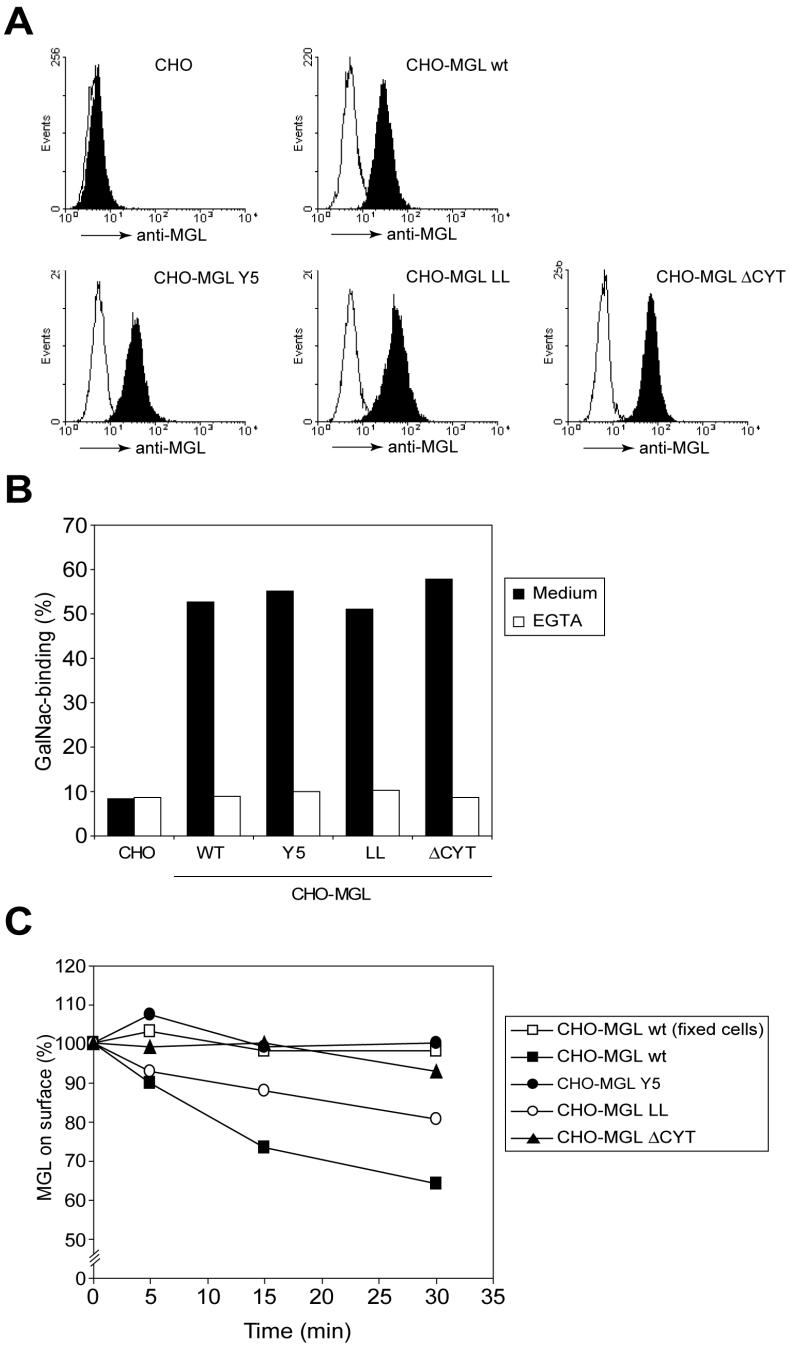

Figure 3. The YENF motif regulates MGL-mediated endocytosis. (A) Expression of wildtype and mutant MGL on CHO-transfectants as measured by flow cytometry. (B) Binding of GalNAc-coated beads to CHO-transfectants of wildtype and mutant MGL. Standard deviation <5% (C) Internalization of cell surface bound antibodies to MGL as measured by flow cytometry on CHO-MGL transfectants. Values represent mean of duplicates. Results are representative for three independent experiments.

antibody to MGL resulted in specific IFNγ secretion by responder T cells at concentrations above 3 ng/ml, demonstrating that ligands that are endocytosed by MGL, can be presented in the context of MHC class II molecules (Fig. 4B). More efficient antigen presentation compared to MGL, was seen after antibody targeting to DC-SIGN, which might reflect antibody affinity or more likely, cell surface expression levels, which are a proportional 10-fold higher for DC-SIGN (Fig. 2A). Isotype control antibodies (anti-ICAM-2) did not induce specific IFNγ secretion. Thus, we conclude that MGL functions as an efficient antigen receptor on DCs.

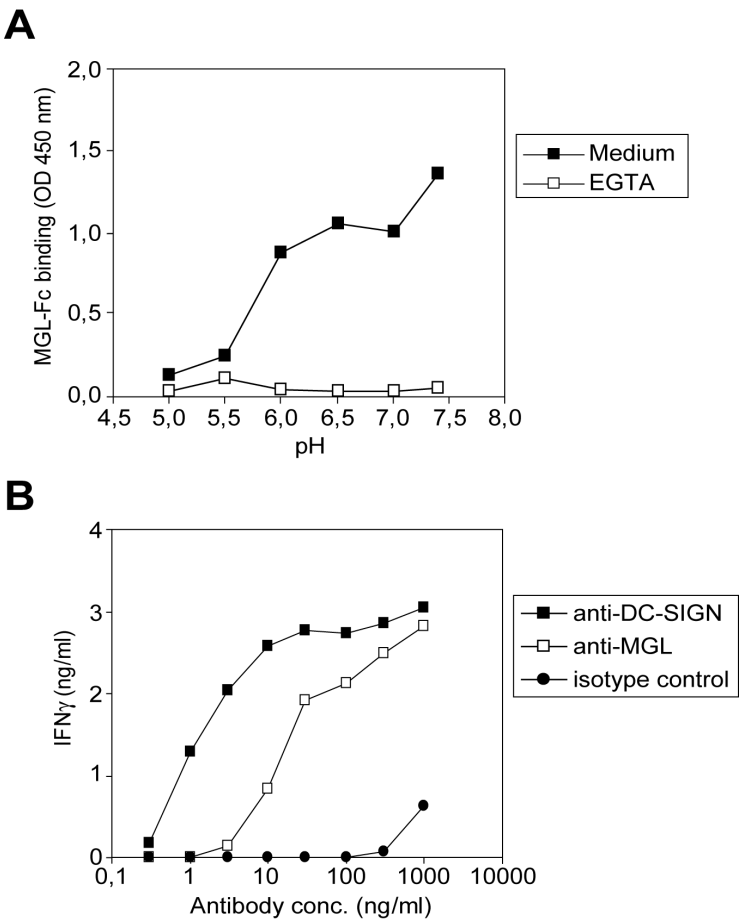

Figure 4. MGL functions as an antigen receptor on human DCs. (A) MGL-Fc binding to GalNAc-PAA was measured by ELISA at indicated pH values. One representative experiment out of three is shown. (B) Proliferation of IgG1-specific T cells induced by immature DCs was analyzed in the presence of serial dilutions of anti-MGL (MLD-1), anti-DC-SIGN (AZN-D1), or anti-ICAM-2 (12A2, isotype control) antibodies. One representative experiment is shown.

DISCUSSION

Here we demonstrate that the human C-type lectin MGL functions as a genuine antigen uptake receptor on DCs. Similar to DC-SIGN, MR and Dectin-1, MGL is capable of targeting soluble ligands for degradation and presentation on MHC class II molecules, resulting in activation of responder T cells[5-7]. It will be interesting to pursue whether endocytosis via MGL leads to cross-presentation of antigens in MHC class I as well.

By mutational analysis, we have shown that the YENF motif in the MGL cytoplasmic tail is crucial for this process. Disruption of the tyrosine-5 residue in this YXXØ consensus motif completely abrogated MGL-mediated endocytosis, whereas

mutating the dileucine motif that only partially matches the consensus motif, only slightly reduced internalization. The YXXØ motif facilitates chlathrin-mediated endocytosis, through the direct interaction of the adaptor protein AP-2[27,32]. Subsequently, clathrin is recruited and endocytosis is triggered. Clathrin-coated vesicles are uncoated after entry and then fuse with the early endosomes[33]. The YXXØ motif has also been implicated in lysosomal targeting, whereby acidic amino acids at the X positions favour lysosomal sorting[27]. The presence of an acidic glutamic acid (E) at position two of the MGL YENF motif indicates that MGL might direct proteins for lysosomal destruction. However, compared to DC-SIGN that dissociates in the late endosomal/lysosomal compartments[5], MGL releases its cargo at higher pH, thus at an earlier stage in the endocytic pathway, likely in early/late endosomes. Therefore, it seems unlikely that MGL travels together with its antigen to the lysosomes. Since the internalization route is also partly dependent on the antigen[8], ligand-induced transfer to the lysosomes cannot be excluded. A similar YXXF motif is present in the cytoplasmic tail of the human transferring receptor[34], which continuously recycles between the plasma membrane and the endosomal compartments. Since MGL mostly resides intracellular[30] and already dissociates from its ligands at pH<6, it might similarly recycle to the cell surface. However, we have not been able to demonstrate any steady state or ligand-induced recycling of MGL (data not shown). Although we observed no major effect of disruption of the dileucine motif on MGL-mediated internalization, we cannot rule out that this motif is involved in the regulation of subsequent intracellular cargo transport.

The tumor-associated Tn antigen (α-GalNAc-Ser/Thr), a high affinity ligand for MGL, is highly expressed by a variety of adenocarcinomas. Generally, Tn expression positively correlates with tumor aggressiveness and early death of patients[35]. *In vitro* and *in vivo* studies have shown that Tn antigens can be presented in the context of MHC class I, resulting in the generation of functionally competent effector T cells[36,37]. Moreover, anti-Tn antibodies are raised in carcinoma patients, proving that Tn epitopes elicit humoral responses and it is therefore an interesting tumor vaccine candidate. The efficacy of such a vaccine is currently being tested in several clinical trials[38,39]. Our data demonstrates that MGL functions as an efficient antigen receptor on DCs, capable of delivering soluble antigens for processing and presentation. The high affinity binding of MGL to Tn epitopes on tumor antigens suggests that *in vivo* MGL may actively participate in stimulating anti-tumor immune responses. This hypothesis is supported by the data obtained in a murine model of ovarian cancer. In this model mMGL$^+$ cells specifically home to the metastatic tumor site and injection of blocking mMGL-antibodies increases metastatic tumor loads, suggesting that MGL positively contributes to anti-tumor immunity[40,41]. However, MGL levels are increased on human DCs with a tolerogenic phenotype[21], indicating that presentation of tumor antigens by these subsets might result in the induction of regulatory T cells or T cell unresponsiveness, thereby promoting tumor growth instead of preventing expansion.

In conclusion, the human DC-expressed C-type lectin MGL internalizes antigens for

presentation to T cells. Future studies are needed to address whether MGL would be useful as a target in tumor-associated glycan-based immunotherapy.

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CHAPTER 7

THE C-TYPE LECTIN MGL EXPRESSED BY DENDRITIC CELLS DETECTS GLYCAN CHANGES ON MUC1 IN COLON CARCINOMA

Eirikur Saeland, Sandra J. van Vliet, Malin Bäckström, Venice C.M. Broks-van den Berg, Teunis B.H. Geijtenbeek, Gerrit A. Meijer and Yvette van Kooyk

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ABSTRACT

The epithelial mucin MUC1 is a high molecular weight membrane glycoprotein frequently overexpressed and aberrantly glycosylated in adenocarcinoma. Mucins normally contain high amounts of O-linked carbohydrate structures that may influence immune reactions to this antigen. During malignant transformation, certain glyco-epitopes of MUC1, such as Tn antigen, TF antigen and their sialylated forms become exposed. The role of these glycan structures in tumor biology is unknown, but their presence is known to correlate with poor prognosis in several adenocarcinomas. We analyzed the potency of MUC1 containing Tn antigens (MUC1-Tn) to target C-type lectins that function as carbohydrate recognition and uptake molecules on dendritic cells (DCs). We identified the macrophage galactose-type lectin (MGL), expressed by both DCs and macrophages, as the receptor for recognition and binding of MUC1-Tn. To validate the occurrence of MGL-MUC1 interactions *in situ*, we studied the binding of MGL to MUC1 in primary colon carcinoma tissue. Isolation of MUC1 out of colon carcinoma tissue showed strong binding activity to MGL. Interestingly, MGL binding to MUC1 was highly correlated to binding by the lectin *Helix pomatia* agglutinin (HPA), which is associated with poor prognosis in colorectal cancer. The detection of MGL positive cells *in situ* at the tumor site together with the modified glycosylation status of MUC1 to target MGL on DCs suggests that MGL positive antigen presenting cells may play a role in tumor progression.

INTRODUCTION

The epithelial mucin, MUC1, is a high molecular weight membrane glycoprotein, normally expressed on the apical surfaces of glandular epithelial cells. It is frequently overexpressed and aberrantly glycosylated in adenocarcinomas, such as breast, pancreas, and colon carcinoma[1-3]. MUC1 is believed to provide a protective barrier, preventing adherence of pathogens as well as lubricating the epithelial layer[4]. More recently, MUC1 has been found to be expressed on activated T lymphocytes[5] and mature dendritic cells[6,7]. The function of MUC1 on leukocytes is currently unknown, although it has been suggested to function in T cell migration[8].

Mucins are heavily glycosylated molecules. The extracellular part of the protein is composed of around 25-100 tandem repeats, each containing 20 amino acids. Within each tandem repeat there are 5 potential O-glycosylation sites that carry glycans, and near the membrane proximal region there are a total of 5 potential N-glycosylation sites[9]. These glycans may comprise ~80% of the molecular weight[1]. During malignant transformation, MUC1 expression is highly upregulated on cancer cells[4,10] and the glycans are severely truncated, resulting in reduction of the total amount of carbohydrates present[11,12]. Both the peptide backbone and new glycan epitopes become exposed to the immune system. The glycan epitopes include Tn (α-GalNAc-Ser/Thr), Thomsen-Friedenreich (TF, Galβ1-3GalNAcαSer/Thr, core 1) antigens and

their sialylated counterparts[13-15]. Expression of these glycan antigens is often increased in colon cancer, whereas they are not expressed in normal colon[16,17]. Such changes are particularly useful when considering diagnostic markers for cancer. For instance, lectin from the Roman snail *Helix pomatia* (*Helix pomatia* agglutinin, HPA) can be used as a prognostic indicator for several adenocarcinomas. HPA interacts specifically with Tn antigens and HPA tissue staining has been shown to correlate with unfavourable prognosis in colorectal[17], breast[18], and gastric carcinomas[19]. However, little is known on the biological effects of modified glycosylation on the immune system and tumor clearance.

Dendritic cells (DCs) play a key role during the initiation of immune responses. Immature DCs internalize antigens for processing and presentation, which often requires specific receptors, such as pattern recognition receptors (PRRs). Immature DCs express various C-type lectin receptors (CLRs), that function as PRRs, and are involved in antigen recognition, uptake and presentation[20]. CLRs specifically recognize carbohydrate structures and engagement of CLRs, such as DC-SIGN[21], mannose receptor[22] and DEC-205[23] is known not to influence DC maturation, and thus antigen uptake through CLRs primarily results in an immunosuppressive or tolerogenic phenotype. However, when DCs properly mature during CLR-mediated antigen uptake the immunosuppressive state of CLRs can be overcome, leading to induction of antigen specific immune responses[24]. This underlines the important role of C-type lectins in steering immune responses. CLRs are likely to be involved in recognition of aberrantly glycosylated tumor antigens, as recently has been shown for the CLR DC-SIGN to recognize specific glycan epitopes on Carcinoma Embryonic Antigen in colon carcinoma[25]. Also for tumor-associated MUC1-glycosylation changes have been observed to influence the growth rate of tumors in a murine model of mammary carcinoma[26] and it interesting to speculate that they may interact with CLRs on DCs, thereby influencing immune responses.

In this study we analyzed the interaction of tumor-associated MUC1 containing Tn antigens (MUC1-Tn) with CLRs on DCs. We identified the macrophage galactose-type lectin (MGL)[27,28] as a receptor for this MUC1 glycoform. Interestingly, MGL bound specifically to MUC1-Tn in primary colon carcinoma and not to MUC1 from normal epithelial cells.

MATERIALS AND METHODS

Cells

Chinese hamster ovary cells (CHO) were cultured in RPMI containing 10% fetal bovine serum (FBS) and streptomycin/penicillin. Monocyte-derived dendritic cells (moDC) were cultured as described before[29], with modifications. In short, human blood monocytes were isolated from buffy coats by a Ficoll gradient step, followed by MACS beads isolation of $CD14^+$ cells (Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytes were cultured in the presence of IL-4 and GM-CSF (500

and 800 U/mL, respectively; ScheringPlough, Brussels, Belgium) for 4 to 6 days. The phenotype of immature DCs was confirmed by flow cytometry ($CD11b^{high}$, $CD11c^{high}$, ICAM-1^{high}, $CD80^{low}$, $CD83^{low}$, $CD86^{low}$). The DCs were matured by incubation with lipopolysaccharide (LPS, *Salmonella typhosa*, 100 ng/mL, Sigma Aldrich, St. Louis, Mo). DC maturation was confirmed by CD80, CD83 and CD86 expression and DC purity was >90%.

Recombinant MUC1-Fc fusion protein

MUC1-Fc containing 32 tandem repeats of MUC1 fused with the Fc part of murine IgG2a was produced in CHO-K1 cells as described before[30]. Purified MUC1-Fc glycoprotein was treated first with V. cholerae neuraminidase (Roche) in 50 mM NaAc pH 5.5, 4 mM $CaCl_2$ at 37°C for 16 h, and then with β-galactosidase from bovine testes (Sigma) in 50 mM NaAc pH 4.5 at 37°C for 16 h, to remove sialic acid and galactose, respectively. The integrity of the MUC1-Fc proteins after this treatment, was checked by SDS-PAGE. The resulting glycoform was determined by ELISA using glycan specific antibodies and lectins (see "Binding assay (ELISA)").

The MUC1-Fc protein used for fluorescent labeling was produced in the glycosylation mutant CHO cells Lec3.2.8.1[31], making only GalNAc O-glycans (and high mannose N-glycans), by the core facility "Mammalian Protein Expression" at Göteborg University and purified by metal chelating chromatography using HiTrap chelating HP (GE Healthcare) loading with Co^{2+}. MUC1-Fc was labeled with Alexa 488 according to manufacturer's instructions (Molecular probes). The MUC1-Fc contains only one potential N-glycosylation site[30] that is probably differently glycosylated in Lec3.2.8.1 cells and CHO-K1 cells[31]. In spite of this, high mannose N-glycans did not result in altered binding of MUC1-Fc to DCs (data not shown).

MUC1 samples

Tissue samples from colon carcinoma were collected after surgical removal of the tumor with informed consent of the patients. Primary colon carcinoma tissue and the corresponding normal tissue were obtained from patients from resection specimens, following national and institutional ethical guidelines regarding the use of human tissues. Tissue samples (~0.5-1.0 cm^3) were gently homogenized and incubated in 1-3 ml lysis buffer (TEA buffer containing 1% Triton X-100, 2 mM $CaCl_2$, 2 mM $MgCl_2$ with protease inhibitor cocktail tablets (Roche) depending on the sample volume for 2 days at 4°C under rotation. The lysates were centrifuged at 14000 rpm for 15 min, supernatant collected and stored in aliquots at –80°C. Protein concentration was measured by BCA assay (Pierce, Rockford, IL, USA).

Antibodies and CLR-Fc fusion proteins

The following antibodies were used: clone 214D4 (provided by John Hilkens, Netherlands Cancer Institute, Amsterdam) specifically recognizing the amino acid sequence PDTR in the extracellular domain of MUC1 (glycosylation independent); this antibody was biotinylated using a biotinylation kit (Pierce, Rockford, IL, USA);

DC-SIGN specific antibody AZN-D1[32]; CD68 specific antibody EBM-11; FITC-labeled goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA); LAMP-1 specific antibody (BD Biosciences, Erembodegem, Belgium); peroxidase-labeled goat anti-human Fc (Jackson Immunoresearch); Alexa 488-labeled goat anti-mouse IgG1; Alexa 594-labeled goat anti-mouse IgG2a, and Alexa 594-labeled goat anti-mouse IgG1 (Molecular Probes, Eugene, Oregon). Anti-glycan antibodies 5E5 (MUC1-Tn) and HH8 (MUC1-TF) were provided by Henrik Clausen (University of Copenhagen, Denmark). The MGL-human Fc fusion protein was made in the following way: the extracellular part of MGL (amino acids 61-289) was amplified on pRc/CMV-MGL by PCR, confirmed by sequence analysis and fused at the C-terminus to human IgG1-Fc in the Sig-pIG1-vector[28]. MGL-Fc was produced by transfection of CHO cells and concentration determined by ELISA. An MGL-murine Fc fusion protein was generated by cloning the extracellular part of MGL (including the CD38 signal sequence from the Sig-pIG1 vector) into an pcDNA3 expression vector containing exon 1-3 of murine IgG2a Fc[30].
The 18E4 monoclonal antibody to MGL was generated by immunizing Balb/c mice with purified MGL-Fc. Hybridoma supernatants were screened for the presence of anti-MGL antibodies on CHO-MGL transfected cells.

Beads adhesion assay
Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes, Eugene, OR) were coated with streptavidin as previously described[33]. Streptavidin coated beads were incubated with biotinylated $F(ab')_2$ fragments of goat anti-mouse IgG2a (6 μg/ml, Jackson Immunoresearch) in 0.5 mL PBS/0.5% BSA for 2 hours at 37°C, washed and further incubated with MUC1-Fc fusion proteins at 4°C overnight. Finally, beads were washed, stored in 100 μl PBS/0.5% BSA, and used within one week. The beads adhesion assay was performed as previously described[33]. DCs were incubated with coated beads in Tris-sodium buffer (20 mM Tris-HCl, pH 7, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) with 0.5% BSA and adhesion was determined in the absence or presence of blocking agents (10 mM EGTA, 25 mM monosaccharides (Sigma) or 5 mM mannan) at 37°C for 45 min. Cells were washed and analyzed by flow cytometry.

Binding assay (ELISA)
The solid phase adhesion assay was performed by coating ELISA plates with purified anti-MUC1 antibodies (clone 214D4) in 0.2 M Na_2CO_3, followed by blocking with 1% BSA in Tris-sodium buffer. Negative controls were included for each sample, where no anti-MUC1 antibodies were added. After washing with Tris-sodium buffer, tissue lysates were added (0.2–1.0 mg/ml protein) and incubated at 4°C overnight. Plates were washed and further incubated with MGL-human Fc (0.5 μg/mL) or biotinylated HPA (5 μg/ml, Sigma), dioxygene labeled MAA (5 μg/ml, Boehringer Mannheim) or anti-TF antibodies (clone HH8) for 1 h at room temperature (RT). After washing with Tris-sodium/0.02% tween the binding was

detected by peroxidase-labeled anti-human IgG, -streptavidin (Jackson Immunoresearch), -anti-digoxigenin antibodies or -anti-murine IgG. Specificity was determined in the presence of 10 mM EGTA or 100 mM GalNAc monosaccharides. MUC1 coating was determined by biotinylated anti-MUC1 antibodies (clone 214D4, 2 μg/mL) followed by incubation with peroxidase-labeled streptavidin. The reaction was developed by TMB substrate and optical density measured by a spectrophotometer.

Immunofluorescence analysis and confocal microscopy

Cryosections (7μm) of normal colon epithelium or primary colon carcinoma were fixed in 100% acetone (10 min), washed with PBS, and incubated with first antibody (10 μg/ml) for 1 h at 37°C. After washing, the final staining was performed with Alexa 594-labeled goat anti-mouse IgG2a or Alexa 488-labeled goat anti-mouse IgG1 and nuclear staining was performed with Hoechst. For staining with MGL-murine Fc, cryosections were fixed in 2% paraformaldehyde and all incubation steps were performed in Tris-sodium buffer. The final staining was performed with Alexa 488-labeled goat anti-mouse IgG2a.

To study internalization, Alexa 488-labeled MUC1-Fc (30 μg/ml) was incubated with DCs for 2 h at 37°C. The transferrin receptor was detected by incubating the cells with Alexa Fluor 594-conjugated transferrin (10 μg/ml; Molecular Probes) for 15 min at 37°C prior to fixation. Labeled cells were fixed in 3% paraformaldehyde in PBS and permeabilized in PBS/0.1% saponin prior to staining. Cells were stained with antibodies to LAMP-1, a lysosomal marker, and subsequently with Alexa 594-conjugated anti-mouse IgG1. Next, cells were allowed to adhere to poly-L-lysine coated glass slides and mounted in anti-bleach reagent. Fixed slides were imaged with a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

Statistical analysis

Statistical differences between groups were analyzed by Wilcoxon Matched Pairs test using Graphpad Instat from Graphpad Software, Inc. Correlation was analyzed by a non-parametric Spearman ranks test. Significance was accepted at the $p < 0.05$ level.

RESULTS

Characterization of MUC1-Fc fusion proteins

The extracellular domain of MUC1, including 32 tandem repeats with 5 O-glycosylation sites each, was fused to a murine IgG Fc tail and produced in Chinese hamster ovary cells, as previously described[30]. To obtain a glycoform of MUC1 with mainly Tn antigen, the protein was desialylated and treated with β-galactosidase, leaving only the core GalNAcs on the protein. The resulting MUC1-Tn reacted with antibodies against Tn, but not against TF in ELISA (Fig. 1). These results indicate that the glycosylation mainly consisted of GalNAcs.

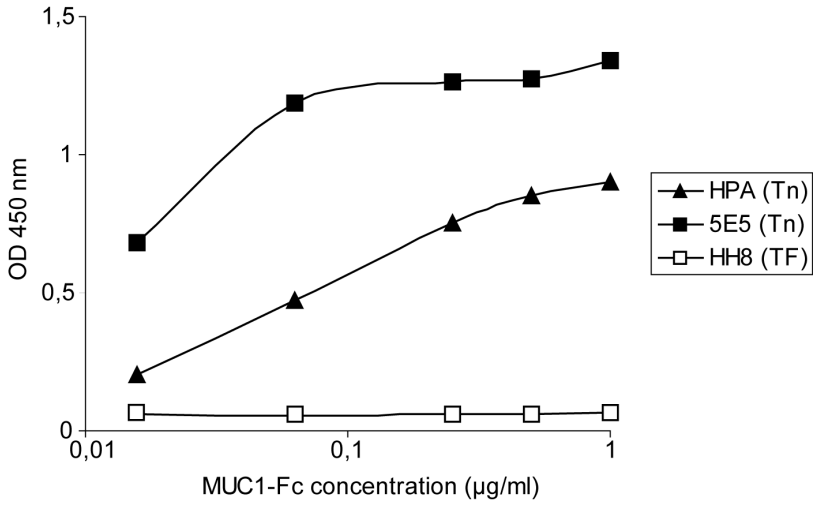

Figure 1. MUC1-Fc fusion proteins contain Tn antigens. MUC1-Fc was serially diluted and coated on ELISA plate. Binding of the following antibodies/lectins was analyzed: Tn-specific antibodies (5E5), HPA (specific for Tn antigens) and TF-specific antibodies (HH8).

MUC1-Fc containing Tn antigens interacts with monocyte-derived dendritic cells

To study the potency of tumor-associated MUC1 to target DCs, MUC1-Fc containing Tn antigens (α-GalNAc-Ser/Thr) was coupled to fluorescent beads and validated for interaction with monocyte-derived DCs (moDCs). Both immature DCs and DCs matured with lipopolysaccharide were analyzed for binding. MUC1-Tn bound efficiently to immature DCs and binding was calcium-dependent, suggesting a role for CLRs (Fig. 2A). Mature DCs showed strongly reduced interaction with MUC1-Tn, concomitant with the fact that mature DCs downregulate expression of CLRs on their cell surface (Fig. 2A). Fluorescent beads coated with polyacrylamide-coupled Lewis X, a carbohydrate epitope for the CLR DC-SIGN[34], served as a positive control. As expected, Lewis X bound strongly to immature DCs that express high levels of

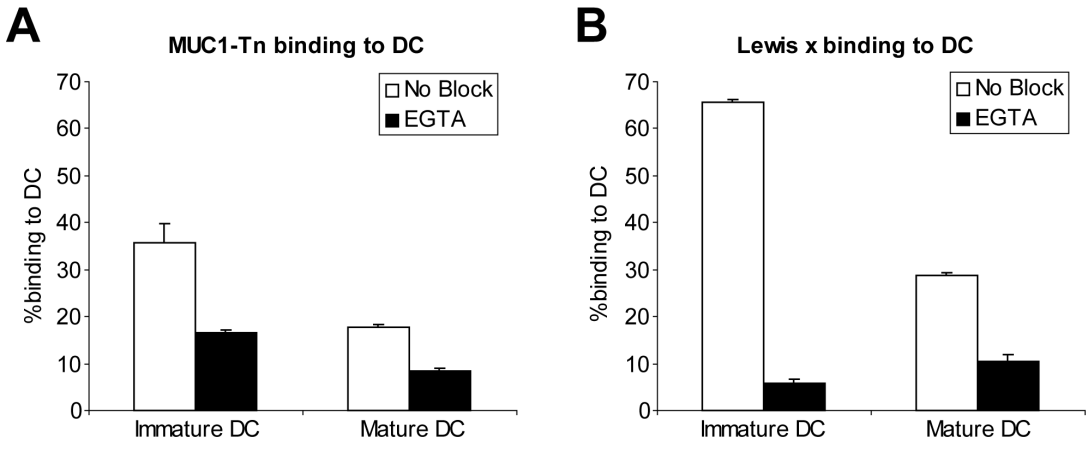

Figure 2. MUC1-Tn interacts with DCs. (A) MUC1-Fc proteins were coupled to fluorescent beads and incubated with day 5 immature or mature monocyte-derived DCs in the presence or absence of 10 mM EGTA. (B) Beads coated with polyacrylamide-coupled Lewis X antigens (ligand for DC-SIGN on DCs) served as a positive control for beads binding. The figure shows the percentage of cells binding beads as determined by flow cytometry. Mature DCs were obtained by incubating DCs in the presence of 100 ng/ml lipopolysaccharide overnight. Samples were analyzed in triplicates (error bars represent standard deviation of the mean) and 1 representative experiment is shown out of 3.

the CLR DC-SIGN and binding to mature DCs, that express low levels of DC-SIGN, was significantly reduced (Fig. 2B). Beads coated only with goat anti-mouse antibodies did not bind to moDCs (data now shown). To determine the specificity of MUC1 binding, DCs were pre-incubated with different carbohydrate monosaccharides (25 mM) to block the interaction with CLRs (Fig. 3A). Only N-acetylgalactosamine (GalNAc) monosaccharides partially blocked binding of MUC1-Tn to DCs, suggesting the involvement of a GalNAc-specific receptor (see below). No block was observed by pre-incubation with galactose, mannose or mannan, suggesting that CLRs such as DC-SIGN or mannose receptor were not involved. Furthermore, MUC1 binding was abrogated after DC maturation, indicating downmodulation of the interacting receptor following DC maturation (Fig. 2A).

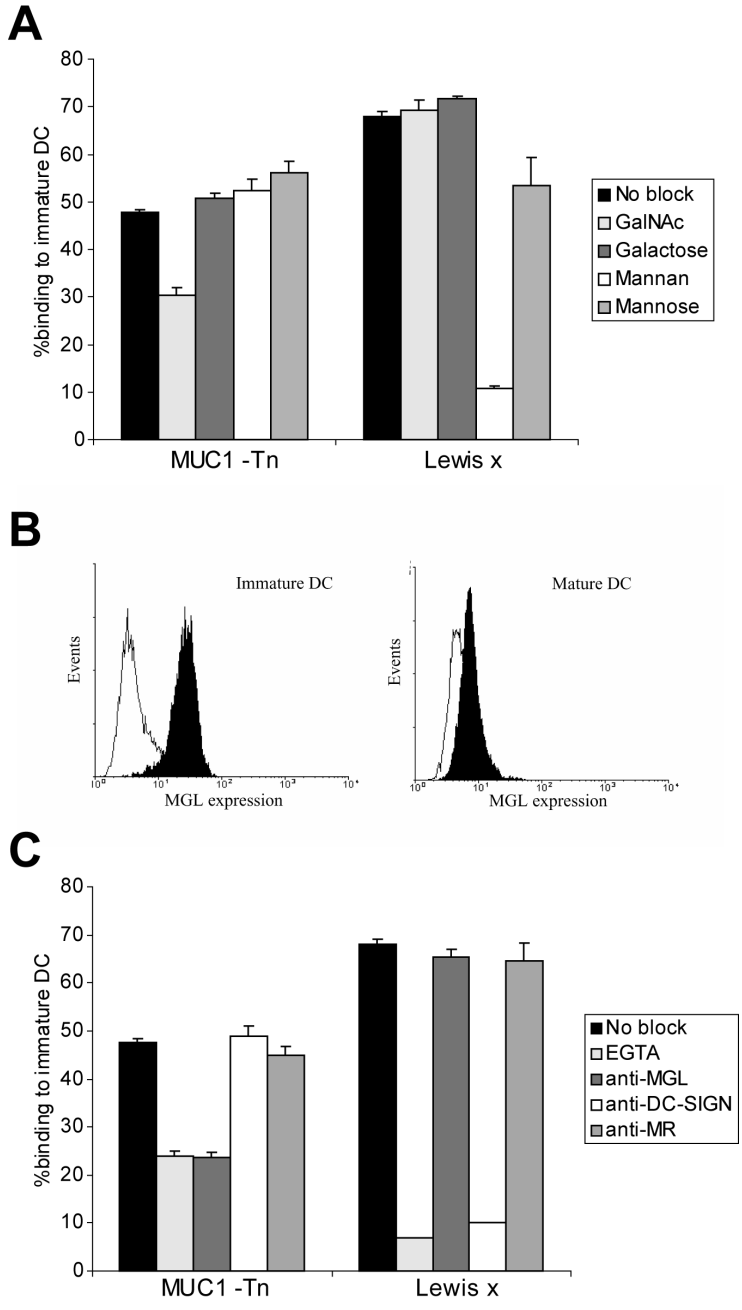

Figure 3. The macrophage galactose-type lectin (MGL) binds to MUC1. (A) To determine the specificity of the MUC1 interaction with DCs, cells were pre-incubated with different saccharides before adding MUC1-Fc coated beads. Only GalNAc reduced binding of MUC1 to DCs and mannan blocked binding of Lewis X. (B) MGL expression on day 5 immature and mature DCs was determined by mAb 18E4. (C) DCs were pre-incubated with different anti-CLR blocking antibodies before incubation with MUC1-Fc coated beads. The figure shows the percentage of cells binding beads as determined by flow cytometry. Samples were analyzed in triplicates (error bars represent standard deviation of the mean) and 1 representative experiment is shown out of 3.

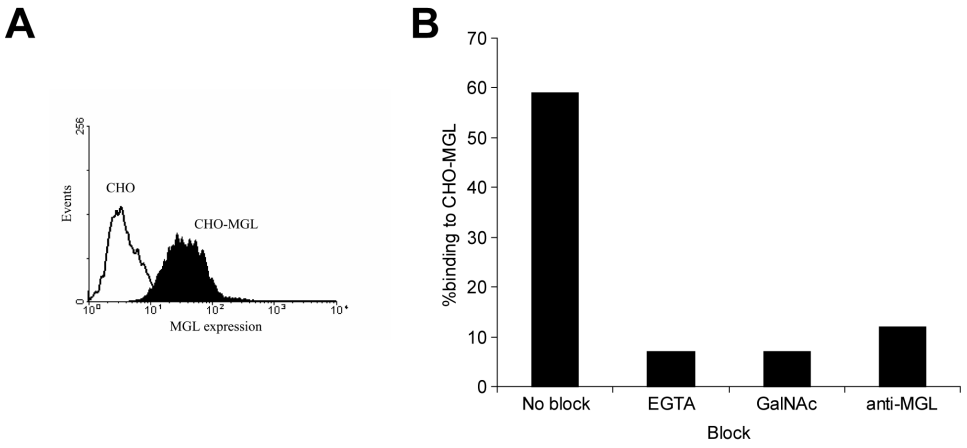

Figure 4. MUC1 binds to MGL transfected CHO cells. (A) MGL expression on CHO cells transfected with MGL (filled histogram). Non-transfected CHO cells served as a negative control. (B) MUC1-Fc-coupled beads were incubated with CHO cells transfected with MGL. Cells were pre-incubated with EGTA or 25 mM GalNAc monosaccharides to block specific interaction with MGL. One representative experiment is shown out of 3.

A candidate C-type lectin, expressed on immature DCs and macrophages, with specificity for GalNAc carbohydrate structures[28,35], is the macrophage galactose-type lectin (MGL)[27]. MGL expression is downmodulated upon DC maturation (Fig. 3B), coinciding with loss of MUC1 binding. Pre-incubation of moDCs with anti-MGL antibodies reduced binding of MUC1-Tn, indicating that MGL is a receptor for this MUC1 glycoform, although the block was not complete. EGTA, which inhibits the

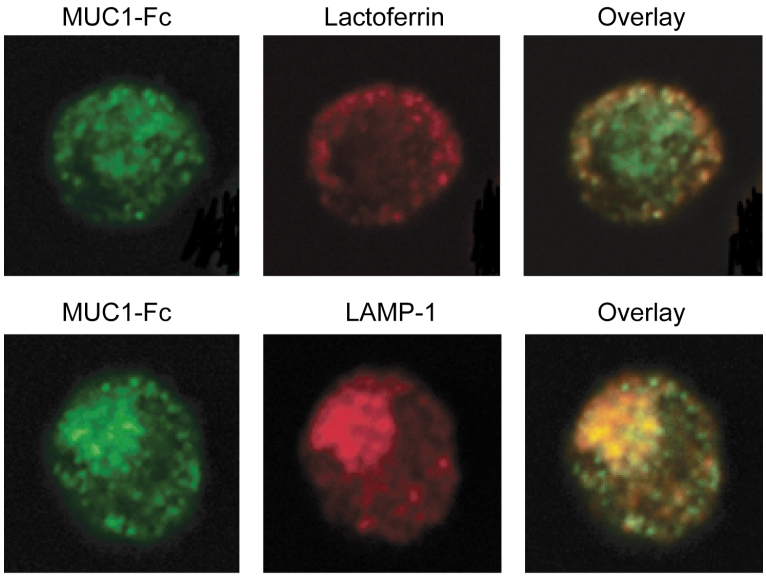

Figure 5. MUC1 co-localizes with LAMP-1 and transferrin after internalization. DCs were incubated with 30 μg/ml Alexa Fluor 488-labeled MUC1-Fc for 2 h at 37°C. Before fixation, the DCs were incubated with Alexa Fluor 594-labeled transferrin for 15 min, which is specifically transferred to the early endosomes. After fixation and permeabilization the DCs were stained with anti-LAMP-1 antibodies (a lysosomal marker), followed by Alexa Fluor 594-labeled goat anti-mouse IgG1.

lectin domain of CLRs to recognize glycans, also inhibited the interaction to the same level as anti-MGL antibodies. We therefore concluded that all CLR binding activity was mediated by MGL (Fig. 3C). Neither anti-DC-SIGN nor anti-mannose receptor antibodies blocked this interaction. The specificity of MUC1-Tn for MGL was confirmed using MGL transfected Chinese Hamster Ovary (CHO) cells (Fig. 4A and 4B). MUC1-Tn bound strongly to MGL transfected cells and interaction was blocked by EGTA, GalNAc, or anti-MGL blocking antibodies, confirming the specificity of the interaction (Fig. 4B). Together, these data strongly indicate that MGL on DCs specifically interacts with tumor-associated MUC1, in particular the Tn antigen.
To study internalization of MUC1, immature DCs were incubated with fluorescently labeled MUC1-Fc for 2 h at 37°C. The cells were also stained for the transferrin receptor (an early endosomal marker) and LAMP-1 (a lysosomal marker). DCs efficiently internalized MUC1 and co-localization was observed with both LAMP-1 and transferrin receptor, suggesting that MUC1 is internalized for lysosomal degradation (Fig. 5). In contrast, the control-Fc protein was not clearly detected in vesicles, but showed diffused staining of much lower intensity.

MGL binds MUC1 derived from primary colon carcinoma

To investigate whether MGL specifically recognizes MUC1 in primary tumors, tissue samples (n=20) were obtained after surgical removal of colorectal tumors from carcinoma patients. The surgical procedure was performed at the VU University Medical Center (VUmc), Amsterdam, the Netherlands and both normal and malignant tissue was obtained from each patient. Total protein concentration was measured in the tissue lysates and found to be significantly higher in the tumor lysates ($p<0.001$) than in normal tissue lysates, probably due to differences in protein content. MUC1 concentration was also significantly higher ($p<0.0001$) in tumor tissue lysates (Fig. 6A and 6B), however, there was no correlation between total protein concentration and concentration of MUC1.
When we analyzed MGL-Fc binding to tumor MUC1, binding was significantly higher than to MUC1 out of normal epithelial tissue from the same patient ($p<0.0001$). Low binding to MUC1 of normal epithelium could be due to low MUC1 concentration in normal colon lysates. Interestingly, however, MGL-Fc binding and MUC1 concentration in tumor samples did not correlate ($r=0.25$; $p=0.28$, Fig. 6A and B) suggesting that there were qualitative differences present that determined MGL-Fc interaction with tumor-associated MUC1. To investigate this further, we analyzed the carbohydrate content of isolated MUC1 from tumor samples and normal epithelial tissues using plant lectins and carbohydrate-specific antibodies. As expected, MUC1 samples varied extensively in carbohydrate reactivity between the different colorectal cancer patients. Some contained Tn antigens, others TF antigens or sialylated structures. Interestingly, a strong correlation was observed between MGL-Fc binding and HPA binding ($r=0.88$; $p<0.0001$), indicating that MGL primarily reacts with Tn antigens on MUC1 in primary colon carcinoma (Fig. 6C). In contrast, no correlation was observed between MGL-Fc binding and the presence of TF

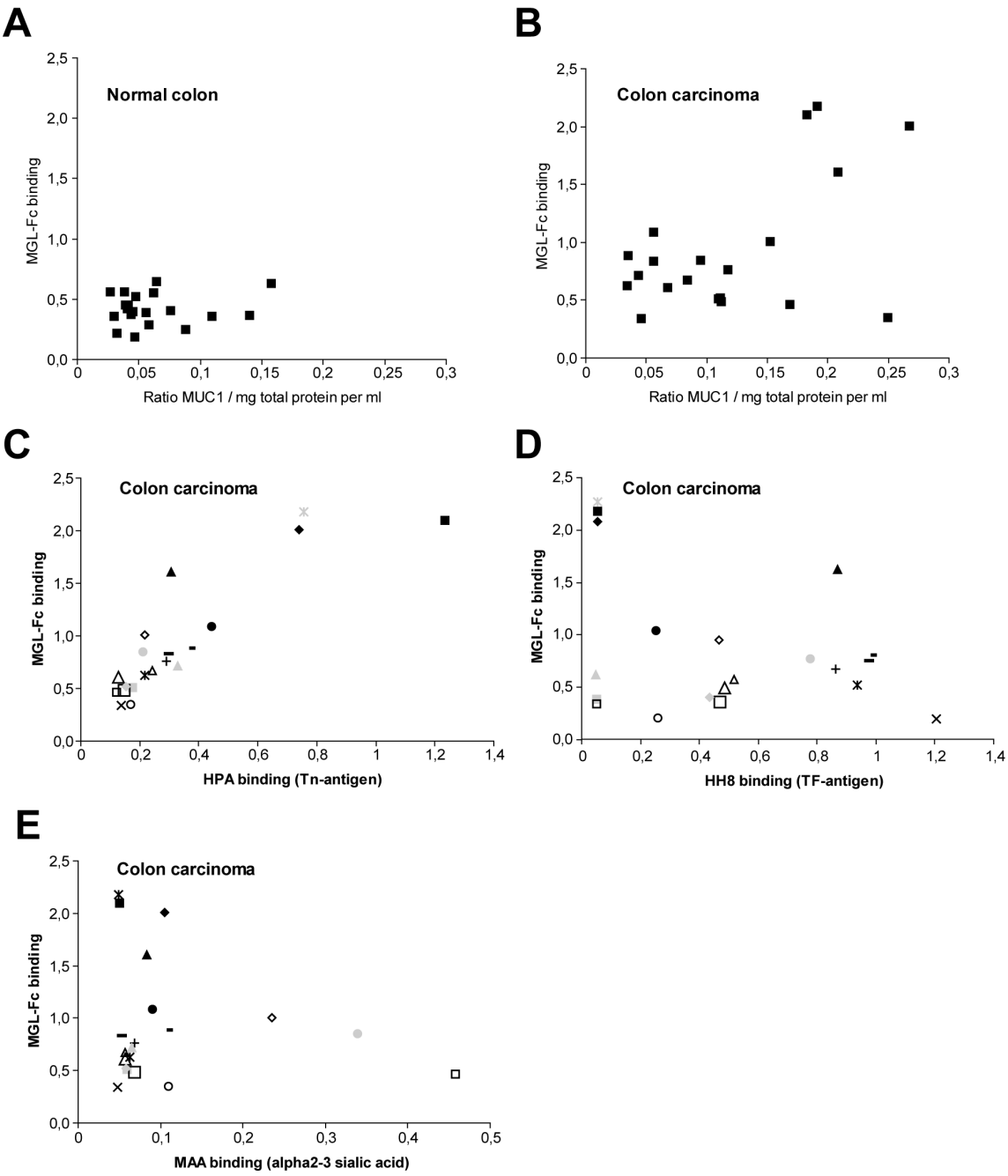

Figure 6. MGL interaction with colon-derived MUC1 mucins. MUC1 was captured from tissue lysates of a primary colon tumor (n=20) and the corresponding normal colon (n=20) by mAb 214D4 in ELISA. The mucins were detected by the same antibody and binding by MGL-Fc, HPA, mAb HH8 and MAA investigated. (A) The relationship between MGL-Fc binding and MUC1 concentration in normal colon lysates. (B) The relationship between MGL-Fc binding and MUC1 concentration in colon carcinoma lysates. Relationship between MGL-Fc and (C) HPA binding, (D) HH8 binding and (E) MAA binding. Each patient is represented by one symbol that can be found in panels C-E. Samples were analyzed in duplicates and one representative experiment out of 2 is shown.

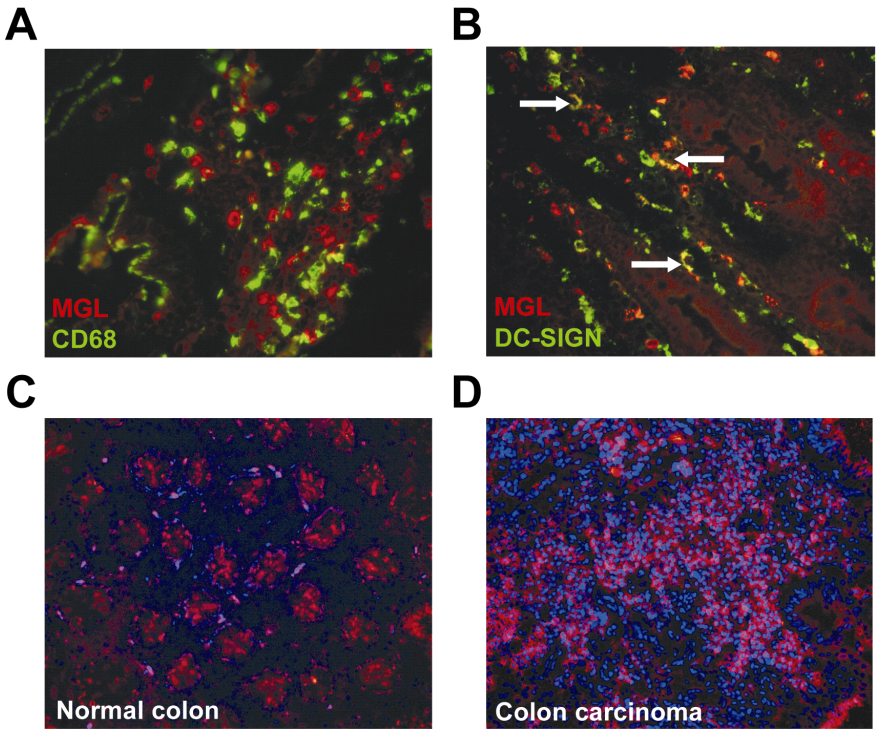

Figure 7. Characterization of MGL expressing cells. Cryosections were stained with anti-MGL (18E4), anti-CD68 (EBM11), anti-DC-SIGN (AZN-D1) or anti-MUC1 antibodies (mAb 214D4) for 2 h at 37°C. After washing the sections were incubated with Alexa 594-labeled goat anti-mouse IgG2a (18E4) and Alexa 488-labeled goat anti-mouse IgG1. (A) Colon carcinoma section stained for MGL (red) and CD68 (green). (B) Colon carcinoma section stained for MGL (red) and DC-SIGN (green). MUC1 staining was performed on both normal tissue (C) and colon carcinoma (D).

antigens or α2-3 sialic acid (Fig. 6D and E). In fact, there was a tendency for inverse correlation, further confirming the data that additional carbohydrate structures would mask the GalNAc epitope interacting with MGL. These data show that both the *in vitro* generated Tn glycoform of MUC1 and the *in situ* tumor-associated MUC1 present in colorectal cancer patients bind strongly to MGL on immature DCs.

MGL expressing cells are detected in the colon

To determine whether MGL expressing cells are present in colon tissue and whether these could interact with MUC1 in tumors, tissue sections were analyzed for cells expressing MGL and MUC1. MGL expressing cells were detected within the normal colonic mucosa of 4 individuals analyzed as well as in primary tumor sections of all these individuals. MGL expressing cells did not appear to express the macrophage marker CD68 in the tumor sections (Fig. 7A), although some co-localization was observed with the CLR DC-SIGN, suggesting that MGL may be expressed on certain DC populations (Fig. 7B).

In normal tissue, MUC1 positive cells were detected at the epithelial layer and some MUC1 positive cells were scattered throughout the mucosa (Fig. 7C). However, as expected MUC1 staining was strong in the tumor tissue (Fig. 7D). The finding that

the MGL expressing cells are located in the vicinity of MUC1 expressing cells demonstrates that MGL positive antigen presenting cells are present in close contact with epithelial tumor cells. To determine whether tumors expressed MGL-binding glycan epitopes, we analyzed the binding activity of MGL-murine Fc to these tissues. MGL-murine Fc showed strong staining of colon carcinoma cells and not with normal colon epithelium (Fig. 8). Furthermore, the fact that the binding of MGL-murine Fc was blocked by GalNAc monosaccharides, demonstrates the requirement of the carbohydrate recognition domain for staining.

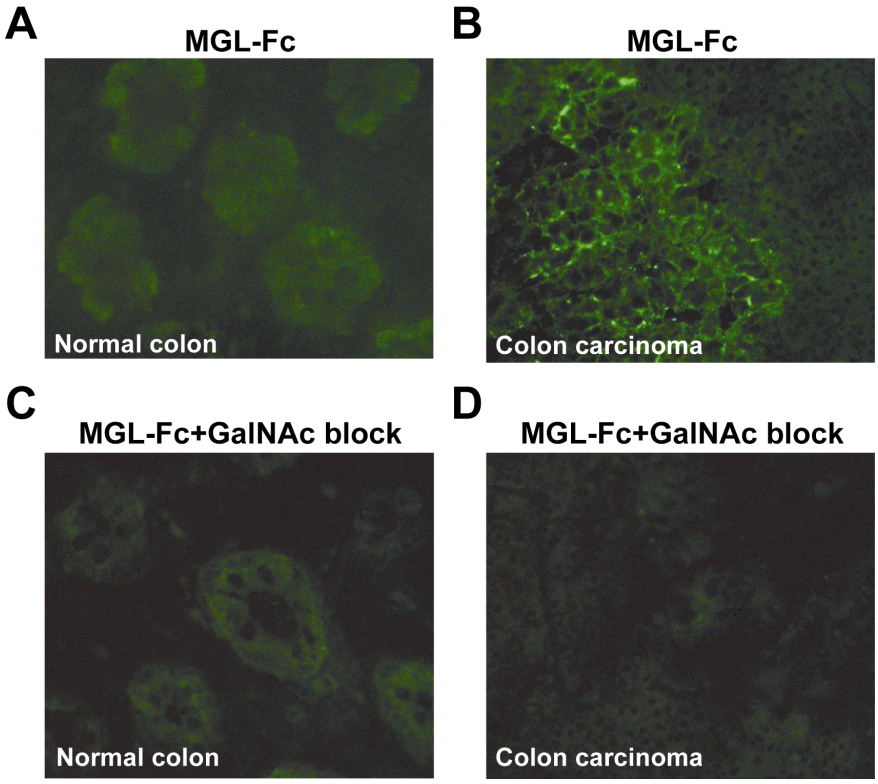

Figure 8. MGL-murine Fc stains colon carcinoma. Cryosections were stained with MGL-murine Fc for 2 h at 37°C, in the absence (A and B) or presence (C and D) of 100 mM GalNAc monosaccharides. After washing the sections were incubated with Alexa 488-labeled goat anti-mouse IgG. Both normal colon epithelium (A and C) and primary colon carcinoma (B and D) were stained from the same patient. Results from one representative patient out of 4 is shown.

DISCUSSION

The epithelial mucin MUC1 is a tumor antigen, whose posttranslational modification is altered during malignant transformation[15]. The protein backbone is retained, but O-linked glycan structures are modified. This creates epitopes that may be recognized by the immune system, including the exposed protein backbone and carbohydrate structures, generally not present in non-malignant tissues. Adenocarcinoma patients may develop humoral and cellular immune responses to the MUC1 protein. These responses are, however, weak and not sufficient to

eradicate the tumor[36,37]. Nonetheless, anti-MUC1 antibody levels have been shown to correlate with survival[38], indicating the importance of this tumor antigen for protection against cancer.

Here we have studied how tumor related MUC1 containing Tn antigens may be optimally targeted to DCs. DCs play a key role in the initiation of immune responses and DC-based tumor vaccines are a promising tool for immunotherapeutic strategies against cancer. Therefore, it is important to understand the molecular mechanisms involved in how tumor antigens are recognized and internalized by DCs to trigger efficient anti-tumor immune responses.

Using recombinant MUC1-Fc proteins containing 32-tandem repeats of the extracellular domain, and a sensitive flow cytometry-based binding assay, we demonstrated that MUC1-Tn bound efficiently to DCs. The interaction was calcium-dependent and was significantly contributed by the CLR MGL, that is expressed on immature DCs, a known receptor for GalNAc containing carbohydrate structures[28,39]. DC maturation resulted in abrogation of MUC1-Tn binding coinciding with loss of MGL expression. We did not observe any involvement of the mannose receptor that has previously been described to be a receptor for glycosylated MUC1[40]. The discrepancy between the two studies is likely due to differences in MUC1 glycoforms. The MUC1 glycoforms studied by Hiltbold *et al* may have contained additional glycan epitopes that target the mannose receptor. The glycoform of MUC1 in our study, primarily contained GalNAc moieties that do not interact with the mannose receptor. We demonstrated MGL-Fc interaction with MUC1 present in tissue samples of primary colon carcinoma patients (n=20) that strikingly correlated strongly with Tn antigen expression (binding by the lectin *Helix pomatia* agglutinin), whereas no correlation was observed with expression of TF antigens or sialylated MUC1. Tn antigens are known to be highly expressed in colon carcinoma, but not in normal colon tissue[17]. Detection of these carbohydrate epitopes by HPA (a GalNAc-specific lectin from the snail *Helix pomatia*) has been shown to be useful for identifying adenocarcinomas with metastatic potential[41,42]. Primary tumors of epithelial origin, which are stained positively with HPA, are more likely to metastasize and this is highly correlated with poor prognosis. It further suggests that these epitopes may play an important role during metastasis[18,43], although it is not clear how this may be achieved or whether there is a role for such structures in tumor cell survival. Interaction of these carbohydrate structures with antigen presenting cells may promote efficient antigen uptake and presentation to T cells. We demonstrated that MUC1 was internalized by DCs and targeted to the lysosomes, which suggests that MUC1 containing Tn antigens may be processed and presented to T cells. However, signaling that occurs during tumor antigen uptake may significantly contribute to the quality of the T cell response. For example, antigen uptake in the absence of Toll-like receptor signaling may result in immunological tolerance[44], so tumor cells may evade immune responses.

We demonstrated the presence of MGL positive cells both within normal colon mucosa and within primary carcinomas, demonstrating that these cells may be in

close contact with the MUC1-Tn expressing tumor cells. Interestingly, these cells seemed to exhibit DC-like phenotype, as they did not express the macrophage marker CD68. MGL-Fc bound strongly to colon carcinoma cells, supporting the hypothesis that MGL positive cells may interact with tumor cells.

Tumor cells may shed MUC1 into the micromilieu and it may be detected in serum of cancer patients[4]. These forms of MUC1 lack a cytoplasmic tail and can (in advanced colon cancer patients) reach such high levels that they can interfere with cell interactions, including selectin interactions[45]. These shed forms of MUC1 thus have the possibility to interfere with the immune system and help the tumor cells escape immune surveillance[45,46]. Interaction with MGL on antigen presenting cells may similarly influence immune responses. It is interesting to mention that MGL is expressed on human immature DCs and in particular highly expressed on tolerogenic DCs and/or macrophages that are cultured in the presence of dexamethasone[47].

In conclusion, our data demonstrate that immature DCs may interact with tumor-associated glycoforms of MUC1 via the CLR MGL. Our finding that MGL binding to MUC1 highly correlates with HPA binding, and the fact that MGL is highly expressed on immature or tolerigenic APCs, suggests that this interaction may lead to immunosuppressive effects. Whether these suppressive effects are due to enhanced antigen uptake, accompanied by a reduced maturation of APCs that leads to the induction of regulatory T cell responses, is currently under investigation.

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CHAPTER 8

N-GLYCOSYLATION OF *CAMPYLOBACTER JEJUNI* PROTEINS INHIBITS DENDRITIC CELL RESPONSES THROUGH THE C-TYPE LECTIN MGL

Nina M. van Sorge, Eirikur Saeland*, Sandra J. van Vliet*, Mijke W. Vogels, Nancy Bleumink, Marc D. Jansen, Jan G.J. van de Winkel, Leonard H. van den Berg, Yvette van Kooyk, W-Ludo van der Pol, Jos P. van Putten

*These authors contributed equally to this work

ABSTRACT

Campylobacter jejuni (*C. jejuni*) infection is a major public health concern. Engagement of pattern recognition receptors (PRRs) on dendritic cells (DCs) is critical for the development of humoral immunity. A unique protein glycosylation (*pgl*) gene cluster regulates N-glycosylation of *C. jejuni* proteins and has recently been identified as a putative virulence factor. Loss of N-linked protein glycosylation in *C. jejuni* (81116::pglA) induced increased DC maturation and cytokine production as compared to the corresponding wildtype strain (81116 Wt). Pgl-dependent glycosylation was required for the interaction with the macrophage galactose-type lectin (MGL), since MGL binding to the pglA mutant strain was completely abrogated. Additionally, transfer of the *pgl* gene locus to E. coli induced binding to MGL. This study demonstrated the *pgl* gene cluster to provide immune modulatory properties to *C. jejuni*, possibly through the C-type lectin MGL.

INTRODUCTION

C. jejuni infection represents the most frequent cause of bacterial enteritis worldwide[1]. Infections are associated with significant mortality and morbidity, especially in young children in developing countries[2]. Clinical symptoms range from loose stools to watery diarrhea or dysentery. Post-infectious (sub-) acute polyradiculoneuropathy (Guillain-Barré syndrome, GBS) is a rare but important sequela of *C. jejuni* infection.

The development of vaccines to prevent *C. jejuni* infections seems feasible, since the incidence of symptomatic *C. jejuni* infections is inversely related with age, due to the development of humoral immunity[1]. Antibody titers are increased up to more than one year after infection[3]. The recent completion of the *C. jejuni* genome sequence[4] has accelerated the identification of virulence factors, for example the identification of a polysaccharide capsule[5-7], and the presence of a unique protein glycosylation (*pgl*) gene cluster, which regulates N-linked glycosylation of *C. jejuni* proteins[8,9]. The *C. jejuni* N-linked glycan was recently identified to be a heptasaccharide containing one D-bacillosamine (D-Bac) and five D-N-acetylgalactosamine (GalNAc) residues with a D-glucose (D-Glc) branch[9]. The *pgl* gene cluster facilitates adhesion of *C. jejuni* to human epithelial cells[10] and colonization of chicks[11], and may therefore represent an important virulence factor in *C. jejuni* pathogenesis.

DCs are believed to represent the first immune cells that interact with *C. jejuni* in the intestinal mucosa. DCs express a repertoire of pathogen recognition receptors (PRRs), which are capable of recognizing or internalizing pathogens[12,13]. C-type lectins represent a subgroup of PRRs, specialized in recognition of specific self and nonself carbohydrate moieties[14]. Several microorganisms have been shown to exploit the function of C-type lectins to interfere with Toll-like receptor (TLR)-induced signaling. For example, the *Mycobacterium tuberculosis* cell wall component ManLAM inhibits DC maturation, interferes with TLR4-induced IL-12 production, and

enhances IL-10 production through interaction with the C-type lectin DC-SIGN (CD209)[15]. The only known receptor with specificity for GalNAc-containing structures is the macrophage galactose-type lectin (MGL)[16]. MGL is expressed on immature DCs[17], IL-4 or IL-13 activated macrophages[18], and during differentiation from monocytes to macrophages[19] The function of this receptor is unknown, although it has been shown to trigger endocytosis of GalNAc glycosylated antigens and interaction with glycosylated tumor-associated antigens[17].
We demonstrate that the *C. jejuni pgl* gene cluster is an immune modulatory virulence factor, which crucially determines DC activation. Additionally, *pgl*-dependent glycosylation was shown to be essential for the interaction with the C-type lectin MGL. Therefore, N-linked glycosylation of proteins may attenuate the primary immune response, through MGL engagement.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *C. jejuni* strains, plasmids and mutants used in this study are listed in table 1. *C. jejuni* strains E98632, P3, GB11, and GB18 were kindly provided by Dr. P. Godschalk (Erasmus Medical Center Rotterdam, The Netherlands). *Campylobacte*r strains were grown under micro-aerobic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C, either on Blood agar base II medium (Oxoid Ltd., London, UK) containing 5% horse blood lysed with 0.5% saponin (Sigma, St. Louis, MO), or in Heart Infusion (HI) broth (Oxoid Ltd). For use in DC activation assays, bacteria in the logarithmic growth phase were harvested from HI broth by centrifugation (5000 x g, 20 min), washed in RPMI without phenol red (Invitrogen, The Netherlands), and diluted to an OD of 1.0 (550 nm). Bacteria were heat-inactivated at 56°C for 1 h. *Escherichia coli* (*E. coli*) strains were grown in Luria-Bertani medium at 37°C (Table 1). When appropriate, the growth medium was supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

In vitro generation and culture of human DCs

Human DCs were generated from peripheral blood monocytes as described previously with minor modifications[32]. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood from healthy volunteers using density-gradient centrifugation over a Ficoll gradient (Amersham Biosciences, Uppsala, Sweden). Recovered PBMC fractions were washed three times in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS). Next, monocytes were purified from the PBMC fraction using density gradient centrifugation over a three-layer Percoll gradient (60%, 47.5% and 34% Percoll in RPMI 1640/10% FCS). Monocytes were harvested from the upper interface and washed three times in RPMI 1640/10% FCS medium, and left to adhere in 6-well plates (4 ml per well, 0.5 x 10^6 cells/ml) for at least 1 h at 37°C. Finally, monocytes were washed twice in RPMI 1640/10% FCS medium and incubated in RPMI 1640/10%

FCS medium supplemented with 2.4 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml human recombinant IL-4 (Strathmann Biotec AG, Hamburg, Germany), and 500 U/ml human recombinant GM-CSF (Leucomax® (molgramostim), Novartis, Basel, Switserland). After six days, immature DCs (iDCs) were harvested, washed and resuspended in RPMI 1640/10% FCS medium for subsequent use.

Strain or plasmid	Genotype or relevant characteristic	Reference
***C. jejuni* strains**		
81116	Wildtype Penner 6 serotype	[20]
81116 Δ*pglA*	81116 *pglA*::Kan	[21]
81116 Δ*rpoN*	81116 *rpoN*::Kan	[22]
129108	Wildtype	[23]
129108 Δ*kpsM*	129108 *kpsM*:Kan	
MK104 (ATCC 43446)	O:19	[24]
E98632	O:6, O:59	[25]
P3 (NTCT 12502)	O:3	
CT28 (ATCC: 49301)	O:63	[26]
GB11	O:2	[25]
GB18	O:19	[25]
HB95-29 (ATCC:BAA-527)	HS:19	[27]
INP7 (ATCC:BAA-528)	HS:19	[27]
INP59 (ATCC: BAA-529)	HS:41	[27,28]
INP21 (ATCC: BAA-530)	HS:41	[28]
***E. coli* strains**		
DH5α	F'/ *endA1 hsdR17subE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF)U169* (Φ*80dlacZ* ΔM15)	[29]
Plasmids		
pBR322	Cloning vector	[30]
pBTLPS	pBR322 containing *pgl* locus from *C.jejuni* 81116	[31]

Table 1. Bacterial strains and plasmids used in this study.

Maturation of DCs by C. jejuni

iDCs were resuspended at a concentration of 0.5 x 10^6 cells/ml in RPMI 1640/10% FCS, and co-incubated with heat-inactivated *C. jejuni*, at a multiplicity of infection (MOI) of 50. Unstimulated DCs served as a control in all experiments. Supernatants were harvested after 24 h and stored at -80°C until further use. DCs were analyzed for expression of maturation markers using flow cytometry.

Flow cytometric analysis of cell surface markers

Surface expression of DC maturation markers was assessed by flow cytometry.

Stimulated DCs were harvested, washed in RPMI 1640/10% FCS, and resuspended in PBS containing 0.1% sodium azide and 0.1% BSA (FACS buffer). Next, cells were incubated with the following antibodies for 30 min at 4°C: FITC-conjugated anti-human HLA-DR (IgG1), PE-conjugated anti-human CD86 (IgG2b), FITC-conjugated anti-human CD83 (IgG1), and PE-conjugated anti-human CD40 (IgG1), or appropriate fluorochrome-labeled isotype controls (all from Becton Dickinson, San Jose, CA). Cells were washed twice using FACS buffer and analyzed using flow cytometry (FACScan, Becton Dickinson).

Measurement of cytokine production

IL-6, IL-10, and IL-12p70 concentrations were determined in harvested supernatants of stimulated DCs using ELISA OptEIA kits according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). TNFα concentrations were quantified using a bioassay with the TNFα-sensitive cell line WEHI 164 (ATCC CRL-1751) as described[33]. WEHI 164 (ATCC CRL-1751) cells were cultured using RPMI 1640, 10% FCS, 250 U of penicillin, and 250 μg streptomycin. Twelve concentrations of human recombinant TNFα (Roche Diagnostics, Mannheim, Germany) were used to construct a TNFα calibration curve.

Isolation and expression of cDNA encoding MGL and MGL-Fc

The cDNA encoding human MGL[16] was amplified from total RNA from immature DCs, using the primers 5'-ATG ACA AGG ACG TAT GAA AAC TTC C-3' and 5'-TCA GTG ACT CTC CTG GCT GG-3', and cloned into the expression vector pRc/CMV. Stable CHO/K1 transfectants were generated using lipofectamin (Invitrogen). MGL positive cells were sorted using MoFlo (DAKOcytomation, Glostrup, Denmark) and MGL specific antibodies. The extracellular part of MGL (amino acids 61-289) was amplified on pRc/CMV-MGL with proof-reading PCR using primers 5'-GGA TCC ACT TAC CTG TGC CTC CGC CGT GAC TCT CCT GGC TGG TC-3' and 5'-GGA TCC CCA AAA TTC CAA ATT TCA GAG GAC-3', confirmed by sequence analysis, and fused at the C-terminus to human IgG1 Fc in the Sig-pIgG1-Fc-vector[34]. MGL-Fc was produced by transient transfection of CHO cells, and concentrations were determined by ELISA.

Protein analysis and lectin blotting

Bacteria were grown overnight, washed once, and resuspended in RPMI 1640 without phenol red at an OD of 1.0 (550 nm). Next, bacteria were mixed with sample buffer and incubated for 10 min at 100°C. Bacterial cell lysates were run on 12.5% polyacrylamide gels, blotted on nitrocellulose membranes, and analyzed using Western blotting. Blots were blocked overnight using Tris buffer (pH=7.0) with calcium and magnesium (TSM) containing 4% bovine serum albumine (BSA) (Roche Diagnostics, Mannheim, Germany), washed four times with TSM containing 0.1% Tween (TSMT), and incubated (1 h, 20°C) with either 1 μg/ml HRP-conjugated *Helix pomatia* agglutinin (HPA-PO, a specific ligand for terminal α-linked N-

acetylgalactosamine) (Sigma, St. Louis, MO) or 0.5 μg/ml MGL-Fc in TSMT/1% BSA and HRP-conjugated anti-human IgG (1:2000 in TSMT/1% BSA). Blots were developed using Supersignal® West pico chemiluminescent substrate (Pierce, Rockford, IL), according to the manufacturer's instructions.

MGL-Fc ELISA

ELISA plates (Maxisorp®, NUNC, Roskilde, Denmark), coated with 1 x 10^8 heat-inactivated *C. jejuni* in PBS, were washed twice using TSM, blocked with TSM/1% BSA (2 h at RT), and incubated with 0.5 μg/ml MGL-Fc in TSM/1% BSA for (2 h at RT), in the absence or presence of EGTA (10 mM) or *N*-acetylgalactosamine (GalNAc, 50 mM) (Sigma, St. Louis, MO). After four rinses with TSMT, plates were incubated (30 min, RT) with HRP-conjugated anti-human IgG, washed five times with TSMT, and incubated with TMB substrate (BD Pharmingen™). Results were analyzed with an ELISA reader at 450nm (Biorad, Benchmark microplate reader).

RESULTS

C. jejuni efficiently stimulates DC maturation and cytokine production

Stimulation of iDCs with heat-inactivated *C. jejuni* strain 81116 resulted in increased expression of the costimulatory molecules CD86, CD40, and the DC maturation marker CD83 (Fig. 1A), reaching peak levels after 24 h of stimulation. Maturation

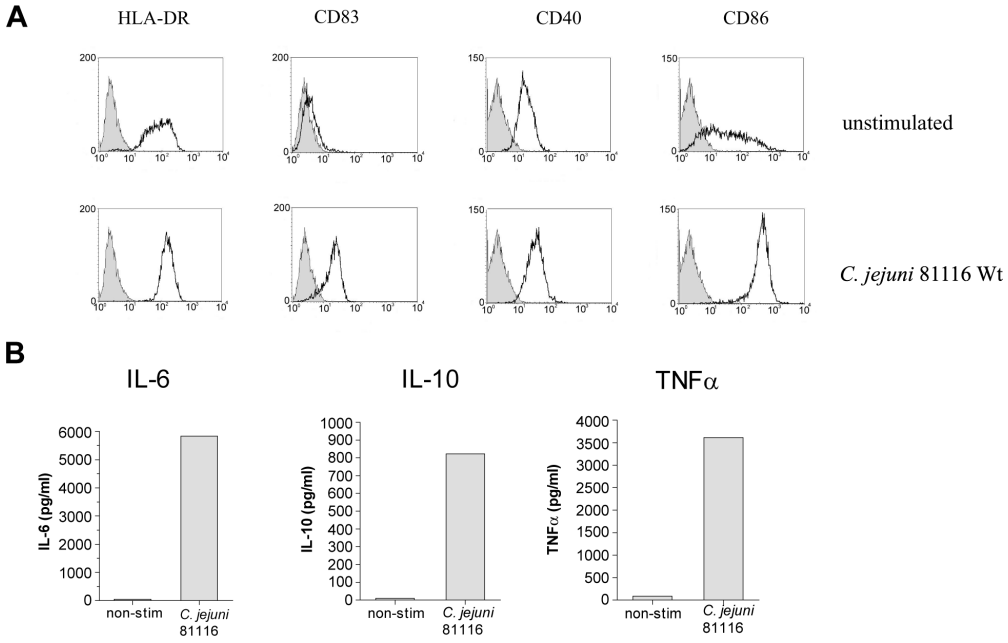

Figure 1. C. jejuni induces DC maturation and cytokine production. iDCs were cultured from peripheral blood monocytes using IL-4 and GM-CSF. iDCs were stimulated for 24 h with *C. jejuni* strain 81116. (A) Expression of maturation markers on *C. jejuni*-stimulated DCs. (B) Production of inflammatory cytokines by *C. jejuni*- stimulated DCs. Results are representative of four independent experiments.

was accompanied by production of IL-6, IL-10, and TNFα, while production of IL-12p70 was generally undetectable or low (Fig. 1B).

Verification of a C. jejuni N-glycosylation deficient mutant

To assess the influence of *N*-linked protein glycosylation on DC activation by *C. jejuni*, a *C. jejuni* strain deficient for the N-linked glycosylation pathway (*pglA*::kan, Table 1) was used. Protein glycosylation patterns of both wild type (Wt), and *pglA* mutant strains were verified by Western blotting with the α-GalNAc-specific lectin HPA[35]. HPA binding to the *pglA*::kan mutant was impaired (Fig 2), consistent with the lack of the *N*-linked GalNAc-glycosylated *C. jejuni* proteins. HPA binding to *C. jejuni* lacking capsular polysaccharide (81116 *kpsM*::kan, data not shown) or the O-glycosylated flagellae (81116 rpo*N*::kan, Fig. 2) was unaltered, indicating that disruption of the *pglA* gene resulted in a selective loss of protein modification by GalNAc-containing glycans.

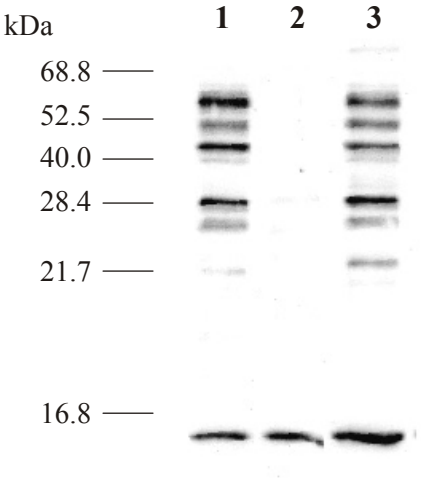

Figure 2. Lack of N-glycosylated proteins in *C. jejuni* pglA mutant strain. Western blot of *C. jejuni* whole cell lysates stained with the GalNAc-specific lectin HPA. Lane 1, 81116 Wt; lane 2, 81116::*pglA*; lane 3, 81116::*rpoN*. Results are representative of three independent experiments.

Effect of N-glycosylation of C. jejuni proteins on DC activation

iDCs were stimulated with heat-inactivated *C.jejuni* Wt 81116 or *pglA* mutant strains, to determine the influence of bacterial N-linked protein glycosylation on DC activation. Loss of N-glycosylated proteins resulted in increased expression of co-stimulatory molecules CD40, and CD86 (Fig. 3A), and enhanced production of IL-6, IL-10, and TNFα (Fig. 3B), compared to stimulation with the parental *C. jejuni* strain. Loss of N-glycosylation did not affect IL-12p70 production (data not shown, n=4).

Interaction of C. jejuni N-glycosylated proteins with C-type lectin MGL

MGL is the only characterized PRR expressed on human iDCs with specificity for GalNAc-containing structures[17]. The presence of multiple GalNAc moieties in N-glycosylated *pgl*-competent *C. jejuni* proteins suggested that this receptor might interact with *C. jejuni* N-glycosylated proteins. Whole cell lysates of *C. jejuni* Wt and *pglA* mutant strains were electrophoresed by SDS-PAGE, blotted on nitrocellulose

membranes, and incubated with MGL-Fc fusion protein. MGL-Fc fusion molecules bound to multiple proteins of the *C. jejuni* Wt strain (Fig 4A, lane 1), while reactivity was completely abrogated when using the *pglA* mutant strain (Fig. 4A, lane 2). The overall binding profiles of MGL and HPA corresponded well, except for a low molecular weight band, possibly representing *C. jejuni* LOS. Binding of MGL-Fc to *C. jejuni* strains with defects in O-glycosylation pathways (81116::*rpoN* (data not shown)) and capsular biosynthesis (81116::*kpsM* (Fig. 4A, lane 4) was similar to Wt 81116.

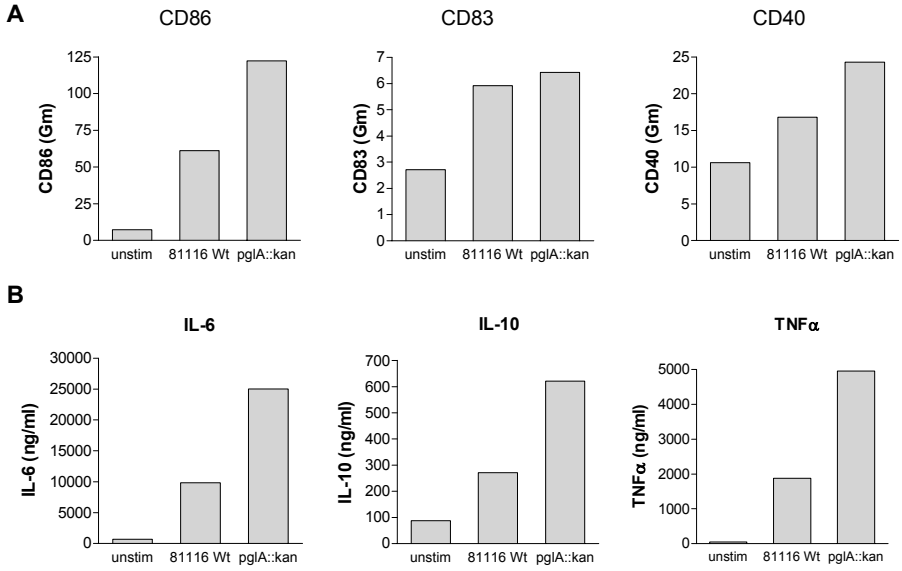

Figure 3. N-linked glycosylation of *C. jejuni* proteins inhibits DC maturation and cytokine production. iDCs were stimulated with heat-inactivated *pglA* mutant or parental 81116 strain for 24 h, and analyzed for (A) expression of DC maturation markers CD40, CD83, and CD86, and (B) production of cytokines IL-6, IL-10, IL-12 and TNFα. Results are representative of five independent experiments.

MGL binding to recombinant E. coli carrying the pgl gene cluster

To verify that the *pgl* gene cluster, indeed, determined binding to MGL, *E. coli* strain DH5α, containing plasmid pBTLPS, carrying the entire *pgl* gene locus of *C. jejuni* strain 81116[31,36], was evaluated next. Presence of terminal GalNAc-containing molecules in the complemented *E. coli* strain was first confirmed by Western blots using HPA (Fig. 4B). Next, whole cell lysates of Wt, control DH5α, carrying the empty vector (pBR322), and *pgl*-complemented DH5α strains were analyzed by Western blotting using MGL-Fc fusion molecules. MGL-Fc stained several proteins bands in DH5α::pBTLPS, which had not been detected using HPA, and were lacking in Wt and control strains (Fig. 4C). Both HPA and MGL-Fc abundantly recognized a low molecular weight band, which may represent GalNAc-containing LPS, as described previously[8].

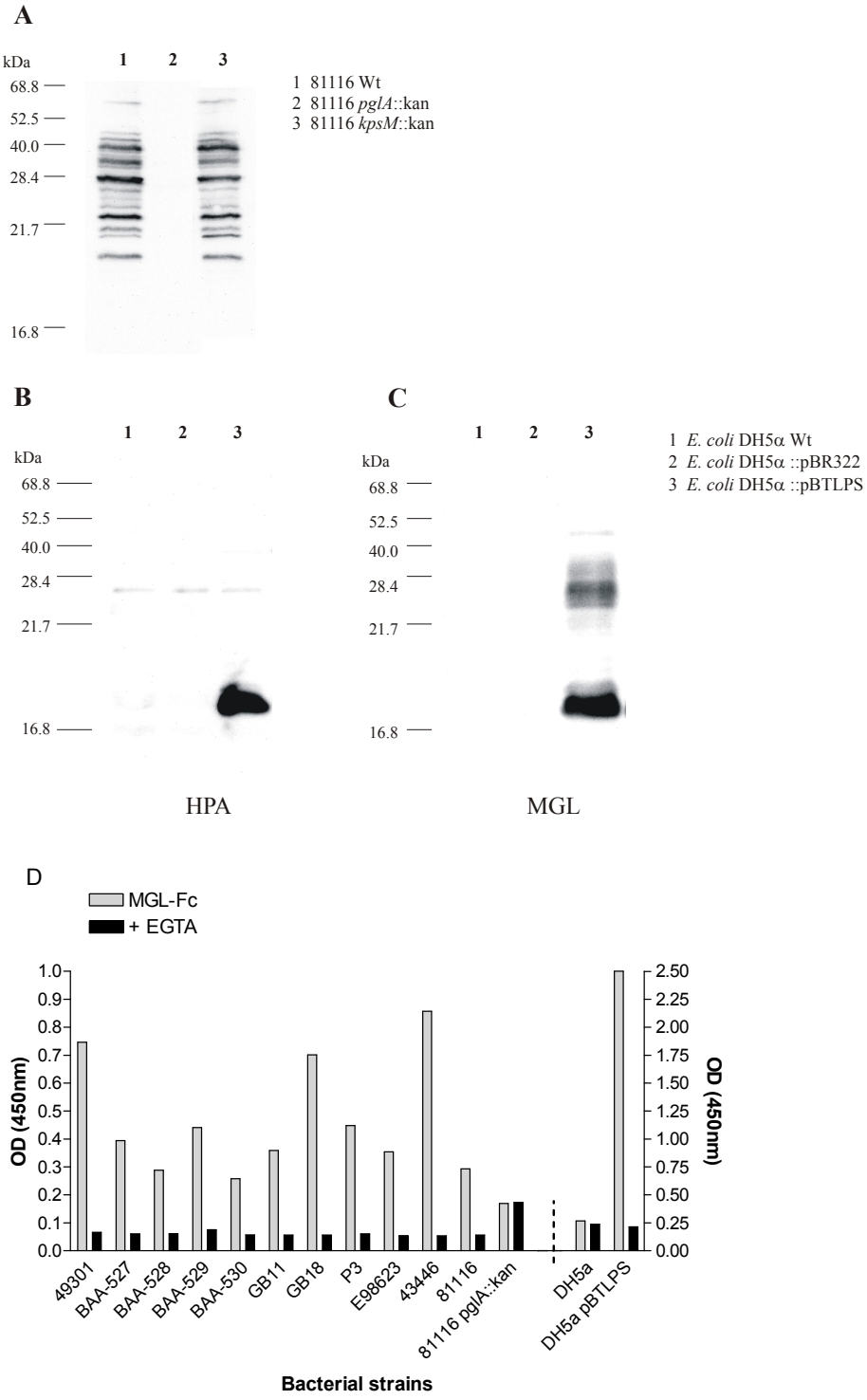

Figure 4. Binding of C-type lectin MGL to N-glycosylated proteins is conferred by pgl gene locus. (A) Western blot of *C. jejuni* whole cell lysates stained with MGL-Fc. Lane 1, 81116 Wt; lane 2, 81116::*pglA*; lane 3, 81116::*kpsM*. HPA staining (B) and MGL-Fc staining (C) of Western blots using *E. coli* whole cell lysates. Lane 1, *E. coli* DH5α Wt; lane 2, *E. coli* DH5α pBR322; lane 3, *E. coli* DH5α pBTLPS. (D) MGL-Fc binding activity to twelve *C. jejuni* strains and Wt and *pgl*-complemented DH5α *E. coli* strains in whole cell ELISA, in the absence or presence of EGTA (10 mM). Results are representative of four (A), or two (B-D) independent experiments.

Conservation of MGL binding activity among C. jejuni strains

To assess whether MGL binding activity was conserved between different *C. jejuni* strains, twelve *C. jejuni* Wt strains were analyzed for their capacity to interact with MGL-Fc molecules in whole cell ELISAs. All tested strains interacted with MGL-Fc, although differences in binding intensity were observed (Fig. 4D). MGL-Fc binding was also observed to the *pgl*-competent *E. coli* strain (Fig. 4D). In contrast, virtually no binding was detectable with the *pglA* mutant or *E. coli* DH5α Wt strain, which served as negative controls (Fig. 4D). MGL binding was abrogated in the presence of EGTA (Fig. 4D) (consistent with calcium-dependent binding of C-type lectins[12]), or free GalNAc (data not shown, n=3).

DISCUSSION

In this study, expression of N-glycosylated proteins by *C. jejuni* was shown to inhibit DC maturation and cytokine production. In addition, *pgl*-dependent glycosylation was found to be required for interaction with MGL, both in *C. jejuni* and a *pgl*-complemented *E. coli* strain. These data strongly suggest that *pgl* genes confer immune modulatory properties to *C. jejuni* through engagement of the C-type lectin MGL on DCs.

Interaction of *C. jejuni* with DCs in the intestinal mucosa might represent the first step in induction of protective immunity, but this has not been studied previously. We demonstrate that *C. jejuni* is capable of inducing DC maturation, as measured by increased expression of costimulatory molecules, and production of cytokines, most prominently IL-6, IL-10, and TNFα. Notably, IL-12p70 expression was generally undetectable or low. Differences in DC activation were observed between DC donors. Functional polymorphisms in PRR[37,38], or in molecules involved in downstream signaling[39], might contribute to these differences.

Since the completion of the *C. jejuni* genome sequence, several putative virulence factors have been identified[4], including four gene clusters involved in carbohydrate biosynthesis[7,40-42]. The *C. jejuni* N-glycosylation cluster, encoded by the 17 kb *pgl* gene locus, regulates the addition of a heptasaccharide to multiple proteins[9,35,43], but not to the polysaccharide capsule or LOS[8,9]. Mutation of the *pgl* gene locus results in diminished immunogenicity[8], reduced adhesive and invasive properties[10], and reduced colonization of chicks[11]. The *pgl* genes may, therefore, play an important role in *C. jejuni* pathogenesis. We confirm that disruption of the *pglA* gene results in significantly reduced protein glycosylation[35], as demonstrated by HPA staining on Western blots. The remaining band on Western blots in *pglA* mutant strain possibly indicates the presence of a terminal GalNAc residue on *C. jejuni* LOS. This LOS carbohydrate modification occurs independent of *pgl* genes, and is subject to phase variation[40,44]. Therefore, the presence of an HPA ligand on LOS can vary between strains and even between mutants and their Wt strains.

Expression of DC maturation markers and cytokine production were increased in response to incubation with the *pglA* mutant, as compared to Wt *C. jejuni*, suggesting

that N-glycosylated proteins interfere with DC activation. In contrast to cytokine production, which was increased in all donors, upregulation of DC maturation markers was detected in only 3 out of 5 donors. The adaptor protein MyD88 was previously shown to be required for the production of inflammatory cytokines by DCs after TLR4 activation, but not for expression of costimulatory molecules[45]. Modulation of DC responses by *C. jejuni* N-glycosylated proteins may therefore be exerted through interference with MyD88-dependent and -independent pathways.

Western blotting of Wt *C. jejuni* demonstrated multiple ligands for MGL. Currently, MGL is the only known receptor expressed on DCs with specificity for GalNAc residues[16,17]. MGL binding was completely abrogated by disruption of the *pglA* gene, indicating that N-glycosylated proteins are MGL ligands. Transfer of the complete *pgl* gene locus to *E. coli* DH5α confirmed that the *pgl* gene locus is responsible for the production of MGL ligands. However, *pgl* genes in *E. coli* mainly seem to modify LPS rather than proteins, as has been demonstrated previously[8,9]. Although binding profiles of MGL and HPA were similar, binding to low Mw bands, possibly representing LPS/LOS, differed. Binding of HPA to *E. coli* and *C. jejuni* LPS/LOS indicates the presence of terminal α-GalNAc moieties, which were not detected by MGL. This differential binding pattern might be explained by the presence of additional sugar moieties or sialic acid residues, which may interfere with MGL binding, but not HPA.

MGL was shown to bind to all *C. jejuni* strains used, although quantitative differences were observed using MGL-Fc whole cell ELISA. It is currently not known whether N-glycosylation patterns differ between *C. jejuni* strains. Such binding diversity may contribute to differences in DC activation.

In conclusion, the present data document immune modulating properties of *C. jejuni* through expression of N-glycosylated proteins via engagement of the C-type lectin MGL on DCs.

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CHAPTER 9

SCHISTOSOMA MANSONI SOLUBLE EGG ANTIGENS ARE INTERNALIZED BY HUMAN DENDRITIC CELLS THROUGH MULTIPLE C-TYPE LECTINS AND SUPPRESS TLR-INDUCED DENDRITIC CELL ACTIVATION

Ellis van Liempt, Sandra J. van Vliet*, Anneke Engering*, Juan Jesus García Vallejo, Christine M.C. Bank, Marta Sanchez-Hernandez, Yvette van Kooyk and Irma van Die

*These authors contributed equally to this work

Molecular Immunology *in press*

ABSTRACT

In Schistosomiasis, a parasitic disease caused by helminths, the parasite eggs induce a T helper 2 cell (Th2) response in the host. Here, the specific role of human monocyte-derived dendritic cells (DCs) in initiation and polarization of the egg-specific T cell responses was examined. We demonstrate that immature DCs (iDCs) pulsed with schistosome soluble egg antigens (SEA) do not show an increase in expression of costimulatory molecules or cytokines, indicating that no conventional maturation was induced. The ability of SEA to affect the Toll-like receptor (TLR) induced maturation of iDCs was examined by co-pulsing the DCs with SEA and TLR-ligands. SEA suppressed both the maturation of iDCs induced by poly I:C and LPS, as indicated by a decrease in costimulatory molecule expression and production of IL-12, IL-6 and TNFα. In addition, SEA suppressed Th1 responses induced by the poly I:C-pulsed DCs, and skewed the LPS-induced mixed response towards a Th2 response. Immature DCs rapidly internalized SEA through the C-type lectins DC-SIGN, MGL and the mannose receptor and the antigens were targeted to MHC class II positive lysosomal compartments. The internalization of SEA by multiple C-type lectins may be important to regulate the response of the immature DCs to TLR-induced signals.

INTRODUCTION

The parasitic helminth *Schistosoma mansoni* is the causative agent of the chronic disease schistosomiasis, which affects ~300 million people worldwide, particularly in tropical countries[1]. The disease is characterized by granulomatous reactions around viable eggs entrapped in host tissues[2]. Schistosomes are multicellular organisms, which present a wide variety of antigens to the host. Increasing evidence indicates that glycoconjugate antigens expressed by the schistosomes play a critical role in the immunobiology of schistosomiasis[3]. Among these are soluble egg antigens (SEA), a complex mixture of diverse glycoconjugates, such as glycoproteins and glycolipids, which are secreted by *Schistosoma mansoni* eggs entrapped in the liver of the host. The early stage of infection with *S. mansoni* leads to a Th1 response. As the infection progresses and eggs are released by the adult worms, the Th1 response declines and switches towards a Th2 response, driven by the egg antigens.
Dendritic cells (DCs) are antigen presenting cells that perform an essential role in the generation and regulation of adaptive immune responses. Precursor DCs migrate from the blood into the peripheral tissues and immature DCs (iDCs) function as a continuous surveillance patrol for incoming foreign antigens. DCs capture and internalize such antigens/pathogens for presentation to T cells in lymph nodes. In addition, DCs provide signals that direct naive Th cells to proliferate and differentiate into Th1, Th2 or T regulatory cells[4]. To become licensed to activate naive Th cells, DCs must undergo a maturation process[5]. Maturation can be induced by immune system intrinsic signals, such as IFNα, TNFα and CD40L[6] or by pathogen-

derived signals[7].
DCs express a wide range of receptors for the recognition of microbes or microbial products, including C-type lectins receptors (CLRs) and Toll-like receptors (TLRs). CLRs recognize carbohydrates on self or nonself glycoproteins. SEA is highly glycosylated enabling its recognition by CLRs. We have previously reported that DC-SIGN[8] and MGL[9] strongly bind SEA, but it is unclear whether other CLRs expressed by DCs are involved in the recognition of SEA and whether the recognition by CLRs leads to internalization of the antigens and induction of Th2 polarizing signals.
Recent reports indicate that schistosome components interact with TLRs. Schistosome egg-derived dsRNA has been reported as a ligand for TLR3[10] and in mice TLR4 has been implicated in Th2 cell development[11]. Lysophosphatidylserine, a lipid from either *S. mansoni* adult worms or eggs, is able to polarize allogenic T cells towards a Th2 development through a TLR2-dependent mechanism[12]. There is increasing evidence that glycans play an important role in the induction of a Th2 response. In mice Lacto-*N*-fucopentaose III, containing the trisaccharide Lewis X, promotes Th2 responses in a TLR4 dependent manner[11,13,14]. Also glycoconjugates carrying complex-type N-glycans with amongst others core α1,3-fucose and core β1,2-xylose determinants, have the capacity to generate a strong Th2-biased cellular response in mice[15]. Such Th2 skewing is seen in many parasite helminth infections and is in general parasitic permissive[16-18], but the Th2 cells also provide the host with protective mechanisms to survive the infection[1]. The mechanisms by which parasite-derived carbohydrates modulate host immune responses remain largely unknown. Certain pathogens that target CLRs through their carbohydrate moieties induce inhibitory or stimulatory signals in DCs that result in modulation of DC function[19-22]. These examples show that CLRs can act in synergy with other receptors, in particular through crosstalk with TLRs, which may also play a role schistosome infection.
To increase our understanding of the role of human DCs in the egg-induced Th2 responses in schistosome infection, we examined in this study the ability of monocyte-derived iDCs, pulsed with SEA or copulsed with SEA and TLR-ligands, to mature and induce polarized T cell responses, and focused on the interaction of SEA with DC-expressed CLRs. Our results show that DC-SIGN, MGL as well as the mannose receptor play a role in binding and subsequent internalization of SEA. Co-localization of SEA with MHC II in the lysosomal compartments suggests that antigen processing and presentation can occur. Although SEA by itself did not induce DC activation, co-pulsing iDCs with SEA and TLR ligands resulted in suppression of the TLR-induced maturation and modulation of the T cell polarizing capacity of DCs.

MATERIALS AND METHODS

Cells

Immature DCs were obtained from buffycoats of healthy donors (Sanquin, Amsterdam) as previously described[23]. In short, human PBMCs were isolated by a

Ficoll gradient. Monocytes were isolated by CD14 magnetic microbeads isolation (MACS; Miltenyibiotec, USA) and differentiated into immature DCs in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Biosource, Nivelles, Belgium). DCs expressed high levels of MHC class I and II, CD11b, CD11c, and ICAM-1 and low levels of CD80 and CD86, as confirmed by flow cytometry

Antibodies and reagents

The following antibodies were used: AZN-D1 (anti-DC-SIGN)[24,25], 1G6.6 (anti-MGL)[26], 3.29.B1 (IgG1, anti-MR)[27], anti-DEC-205[28]; anti-DCIR (R&D systems) anti-LAMP-1 (H4A3, BD Pharmingen), EEA-1 (Abcam, Cambridge, UK), Q5/13 (anti-MHC II)[29], PE-conjugated antibodies against CD80, CD86 and HLA-DR (BD Pharmingen) and CD83 (Immunotech), goat anti-mouse and goat anti-rabbit conjugated with Alexa fluor 594 (Molecular Probes), goat anti-mouse and goat anti-human conjugated with peroxidase (Jackson Immunoresearch, West Grove, Pa). Carbohydrate components mannan, mannosylated biotinylated BSA, GalNAc and laminarin were all from Sigma. Crude *Schistosoma mansoni* soluble egg antigens (SEA) extract was prepared as described previously[30] and was provided by F. Lewis (Biomedical Research Institute, Rockville, MD). SEA does not contain detectable levels of LPS, as was shown by the inability of SEA to induce IL-8 production by TLR2 and TLR4 transfected cell lines (kind gift of Douglas Gohlenbock) (data not shown).

DC maturation

Immature monocyte-derived DCs (day 4) were stimulated with SEA (5 or 50 μg/ml) in the presence or absence of LPS (10 ng/ml; *Salmonella typhosa*, Sigma-Aldrich, St Louise, MO) or poly I:C (10 μg/ml; Sigma-Aldrich) for 24 hrs at 37°C. Analysis of cell surface expression of MHC class II (HLA-DR) and costimulatory molecules CD80, CD83, and CD86 was used to determine maturation.

Cytokine measurements

For the detection of cytokines, culture supernatants were harvested 24 h after DC activation and frozen at -80°C until analysis. Cytokines were measured by ELISA with CytoSets™ ELISA kits (Biosource) for human IL-6, IL-10, TNFα and IL-12p40 according to the manufacturer's instructions. Human IL-12p70 detection was determined as described before[31].

Dendritic cell-driven Th1/ Th2 differentiation

iDCs were cultured from monocytes of healthy donors in Iscove's Modified Dulbecco's Medium (Gibco), supplemented with 10% FCS, 500 U/ml IL-4 and 800 U/ml GM-CSF (Biosource)[23]. At day 6, DC maturation was induced with SEA in combination with LPS or Poly I:C. The following positive controls were included in the assay: (i) 10 ng/ml *E. coli* LPS, (mixed Th1/ Th2 response); (ii) 20 μg/ml poly I:C (Th1); and (iii) 10 μg/ml PGE2 and 10 ng/ml LPS (Th2). After 2 days, DCs were

washed and incubated with autologous $CD45RA^+/CD4^+$ T cells (5×10^3 DC / 20×10^3 T cells). In parallel, DCs were analyzed for maturation markers (CD83 and CD86) by flow cytometry. At day 5, rIL-2 (10 U/ml) was added, and the cultures were expanded for the next 7 days. To determine cytokine production by Th cells, at day 12-15 quiescent T cells were restimulated with 10 ng/ml PMA and 1 μg/ml ionomycin (both Sigma-Aldrich) for 6 h. After 1 h 10 μg/ml Brefeldin A (Sigma-Aldrich) was added to the T cells. Single cell production of IL-4 and IFNγ was determined by intracellular flow cytometric analysis. Cells were fixed in 2% PFA, permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with anti-human IFNγ-FITC and anti-human IL-4-PE (BD Pharmingen).

mRNA isolation and cDNA synthesis

mRNA was isolated by poly (A+) RNA capture in streptavidin-coated tubes with an mRNA Capture kit (Roche, Switzerland) and cDNA was synthesized with the Reverse Transcription System kit (Promega, USA) following manufacturer's guidelines. In brief, cells were washed twice with ice-cold PBS and harvested with 100 μl lysisbuffer. Lysates were incubated with biotin-labeled $oligo(dT)_{20}$ for 5 min at 37°C. The mix was transferred to streptavidin-coated tubes and incubated for 5 min at 37°C. After washing 3 times with 200 μl washing buffer, 30 μl of the reverse transcription mix (5 mM $MgCl_2$, 1x reverse transcription buffer, 1 mM dNTP, 0.4 U recombinant RNasin ribonuclease inhibitor, 0.4 U AMV reverse transcriptase, 0.5 μg random hexamers in nuclease-free water) were added to the tubes and incubated for 10 min at room temperature followed by 45 min at 42°C. To inactivate AMV reverse transcriptase and separate mRNA from the streptavidin-biotin complex, samples were heated at 99°C for 5 min, transferred to microcentrifuge tubes and incubated on ice for 5 min, diluted 1:2 in nuclease-free water, and stored at -20°C.

Quantitative real-time PCR

Oligonucleotides were designed by using computer software Primer Express 2.0 (Applied Biosystems, USA) and synthesized by Isogen Lifescience (IJsselstein, the Netherlands). Primer specificity was computer tested (BLAST, National Center for Biotechnology Information) by homology search with the human genome and later confirmed by dissociation curve analysis. PCR reactions were performed with SYBR green method in an ABI 7900HT sequence detection system (Applied Biosystems, USA). The reactions were set on a 96-well plate by mixing 4 μl of the 2x concentrated SYBR Green Master Mix (Applied Biosystems) with 2 μl of the primer solution containing 5 nmol/μl of both primers and 2 μl of a cDNA solution. The thermal profile for all the reactions was 2 min at 50°C, followed by 10 min at 95°C and then 40 cycles of 15 sec at 95°C and 1 min 60°C. The fluorescence monitoring occurred at the end of each cycle. The Ct value is defined as the number of PCR cycles where the fluorescence signal exceeds the threshold value, which is fixed above 10 times the standard deviation of the fluorescence during the first 15 cycles and typically corresponds to 0.2 relative fluorescence units. This threshold is set constant

throughout the study and corresponds to the log linear range of the amplification curve. The normalized amount of target, or relative abundance, reflects the relative amount of target transcripts with respect to the expression of the endogenous reference gene, GAPDH[32].

Fluorescent bead adhesion assay

SEA binding to whole cells was measured by bead adhesion assay as described previously[33]. In short, streptavidin was covalently coupled onto carboxylate-modified TransFluorSpheres (488/654 nm, 1.0 μm, Molecular Probes Inc, Eugene, OR). SEA was biotinylated with EZ-link™ NHS-LC-LC-Biotin, according to the manufacturer's protocol (Pierce, Rockford, IL). Biotinylated SEA was coupled to streptavidin coated fluorescent beads[34]. Cells were resuspended in TSM (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) and 0.5% BSA. After pre-incubation with blocking antibodies against C-type lectins (20 μg/ml), mannan (50 μg/ml), GalNAc (50 mM), mannosylated biotinylated BSA (50 μg/ml), laminarin (100 μg/ml) or EGTA (10 mM) for 10 min at room temperature in TSM/0.5% BSA, cells were incubated with ligand-coated fluorescent beads (20 beads/cell) for 45 min at 37°C. Bead adhesion to the cells was determined by flow cytometry (FACScan, Becton Dickinson, Oxnard, CA).

Internalization assay

Immature DCs were incubated with biotinylated SEA (10 μg/ml) in TSA/0.5% BSA for 1 h on ice and were subsequently washed. Where indicated cells were pre-incubated with Abs directed against C-type lectins for 30 min at 37°C. Cells were incubated at 37°C for various times, placed on ice and incubated with Alexa fluor 488-labeled streptavidin. To control for off-rate of SEA at 37°C, cells were fixed (2% PFA) before SEA binding to prevent membrane transport. Cells were analyzed using flow cytometry, and the relative difference in mean fluorescence intensity compared with fixed cells was calculated.

Confocal laser scanning microscopy

SEA was labeled with Alexa Fluor 488 according to the manufacturer's protocol (Molecular Probes). DCs were incubated for 2 hrs at 37°C with Alexa Fluor 488-conjugated SEA (10 μg/ml). Labeled cells were fixed and permeabilized for 20 min at 4°C (BD cytofix/cytoperm™, BD). Cells were stained in phosphate-buffered saline containing 0.5 % bovine serum albumin and 0.1% saponin, with antibodies against LAMP-1, EEA-1, DC-SIGN, MGL or MR and subsequently with Alexa Fluor 594-conjugated secondary antibodies. Cells were allowed to adhere to poly-L-lysine-coated glass slides, mounted with anti-bleach reagent and analyzed by confocal microscopy. The Leica AOBS SP2 confocal laser scanning microscope (CLSM) system was used, containing a DM-IRE2 microscope with glycerol objective lens (PL APO 63x/NA1.30) and images were acquired using Leica confocal software (version 2.61).

RESULTS

SEA inhibits DC activation induced by TLR3 and TLR2/4 ligands

To examine the capacity of SEA to induce maturation of human DCs, we pulsed monocyte-derived iDCs with SEA in different concentrations. SEA alone did not induce DC maturation, as none of the activation markers CD80, CD83, CD86 or HLA-DR were upregulated, not even at high concentrations of SEA (up to 100 μg/ml, Fig. 1A and data not shown). Furthermore, iDCs incubated for 24 h with SEA do not secrete any IL-10, IL-6, IL-12p40, IL-12p70 or TNFα, as is shown for 5 and 50 μg/ml SEA in figure 1B. Next, we investigated whether SEA could inhibit the maturation of DCs induced by the TLR3 and TLR2/4 ligands, poly I:C and LPS respectively, to mimic the situation in schistosome infection where TLR2, TLR3 and TLR4 have been implicated to play a role[10-12]. The results show that both the LPS and the poly I:C induced maturation was inhibited in the presence of SEA in a dose-dependent manner, as 50 μg/ml SEA induced more inhibition than 5 μg/ml SEA. CD80, CD83 and CD86 upregulation is inhibited (30 to 45%) compared to LPS- or poly I:C-activated DCs (Fig. 1C). Furthermore, in the presence of SEA, DCs produced reduced levels of all cytokines tested, TNFα, IL-6, IL-10, IL-12p40 and IL-12p70, in a dose-dependent manner (Fig 1D). For IL-10 we observed that although most donors tested (19 out of 30) displayed a reduction in IL-10 production in the presence of SEA, some donors did not produce IL-10 at all (n=3), or produced equal amounts of IL-10 in the presence or absence of SEA (n=8). In conclusion, SEA does not induce maturation of human iDCs, but inhibits both the TLR3 and TLR2/4 ligand induced activation of DCs. Such inhibition is not a generally observed; as for example E/S products secreted from schistosomula do not have this effect on DC activation (data not shown).

Modulation of polarized T cell responses by SEA

In schistosomiasis, the early phase of infection is characterized by Th1 immune responses that are modulated to Th2 upon egg laying, driven by the egg antigens[1]. To assess the DC-driven T cell responses, DCs were pulsed with SEA in combination with LPS or poly I:C and cocultured with naive T cells. As described previously[35], DCs incubated with LPS induce naive T cell differentiation into a mixed Th1/Th2 response, whereas poly I:C induces a dominant Th1 response and PGE2 a Th2 response (Fig. 2). Our data show that SEA alone could not support T cell differentiation (Fig. 1 and data not shown). Interestingly, coculture of iDCs with a mixture of SEA and TLR ligands resulted in a downmodulation of the Th1 responses induced by poly I:C, and skewing of the mixed Th1/Th2 response induced by LPS towards a Th2 response. SEA could not skew the Th1 response of poly I:C towards a Th2, possibly because the Th1 response induced by poly I:C is too strong.

SEA binds to both immature and mature dendritic cells.

To assess the capacity of different immune cells to bind SEA, a bead adhesion assay

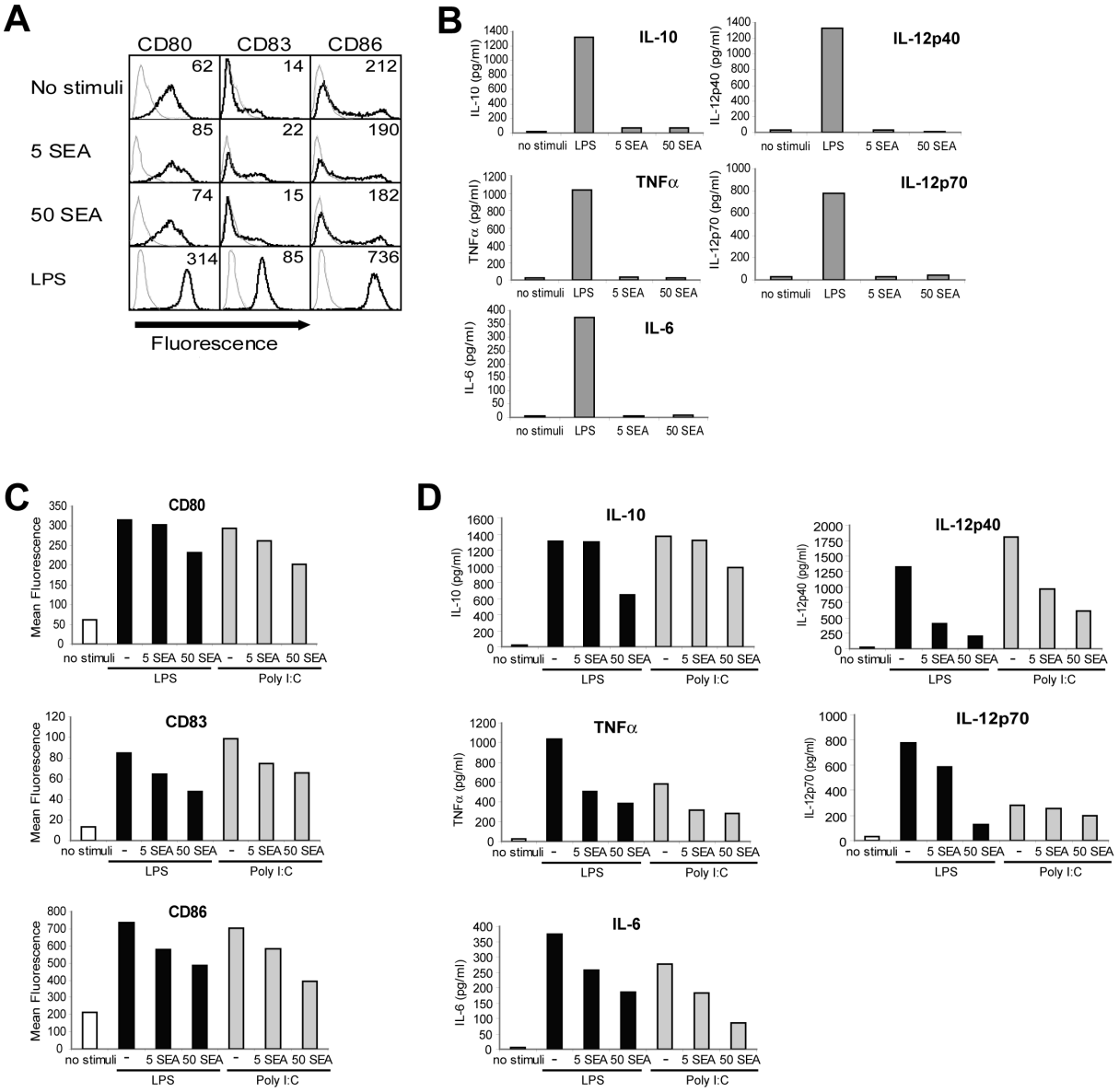

Figure 1. SEA inhibits the poly I:C- or LPS-induced DC activation and cytokine production. (A) SEA does not induce maturation of immature DCs. Immature DCs were incubated with LPS or different concentrations of SEA for 18 h, and activation was determined by measuring the expression of CD80, CD83 and CD86. Dotted line represents isotype controls; the dark line represents immature DCs treated with either LPS (10 ng/ml), SEA (5 or 50 μg/ml). One representative experiment out of three is shown. Numbers reflect mean fluorescence intensity values. (B) SEA does not induce production of IL-10, IL-6, TNFα, IL-12p40 or IL-12p70. Supernatants were harvested after 18 h and cytokine production was measured by ELISA. (C) SEA inhibits the LPS- or poly I:C-induced maturation. Immature DCs were treated with LPS (10 ng/ml) or poly I:C (10 μg/ml) in the presence or absence of SEA (5 or 50 μg/ml). Activation was determined as in A. (D) SEA inhibits the LPS- or poly I:C-induced cytokine production for IL-10, IL-6, TNFα, IL-12p40 and IL-12p70, in a dose-dependent manner. Supernatants were harvested after 18 h and cytokine production was measured by ELISA.

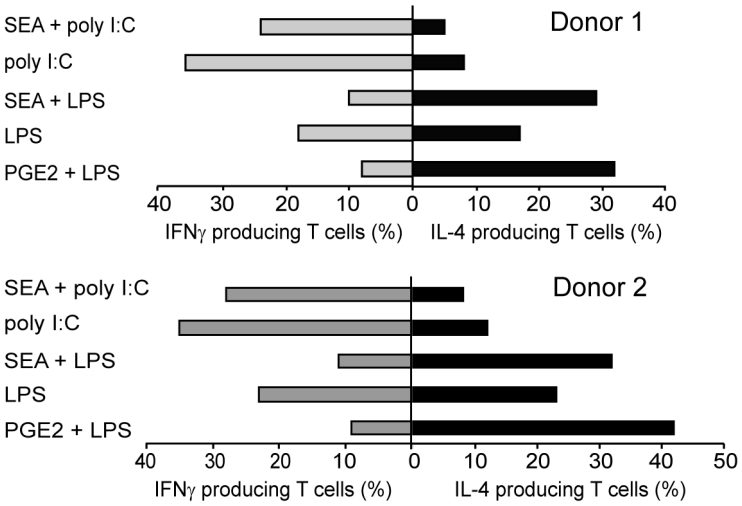

Figure 2. Poly I:C and SEA costimulation results in a down modulated Th1 response. DCs were incubated with SEA, LPS, poly I:C or PGE2 for 48 h, washed and cocultured with highly purified $CD45RA^{+}CD4^{+}$ T cells. Naive T cells were restimulated with PMA and ionomycin, and intracellular IL-4 (Th2) and IFNγ (Th1) was analyzed on a single cell basis by flow cytometry. Representative results of two out of five donors are shown.

was performed. SEA was biotinylated, coupled to streptavidin-coated fluorescent beads[34] and incubated with different immune cells. As shown in figure 3, SEA does not bind to monocytes, PBMCs or PBLs isolated from blood of healthy donors. In contrast, SEA bind strongly to iDCs, and displays a reduced binding to DCs matured with LPS or poly I:C (Fig. 3). The binding of iDCs to SEA could be completely blocked by preincubation with EGTA, indicating that the binding is Ca^{2+}-dependent. Both findings suggest that DC-expressed CLRs are involved in the recognition of SEA.

Expression of pathogen receptors on DCs

We have previously reported that SEA is recognized by iDCs through the CLRs DC-SIGN[8] and MGL[9]. Because SEA contains many different glycan antigens, it is likely that also CLRs other than DC-SIGN and MGL are involved in the binding of SEA. To identify possible candidate lectins, we determined the expression levels of CLRs by RT-PCR for both immature and mature DCs. DCs express high levels of DC-SIGN, MR and MGL as is indicated by a relative abundance of 1 or higher, moderate levels

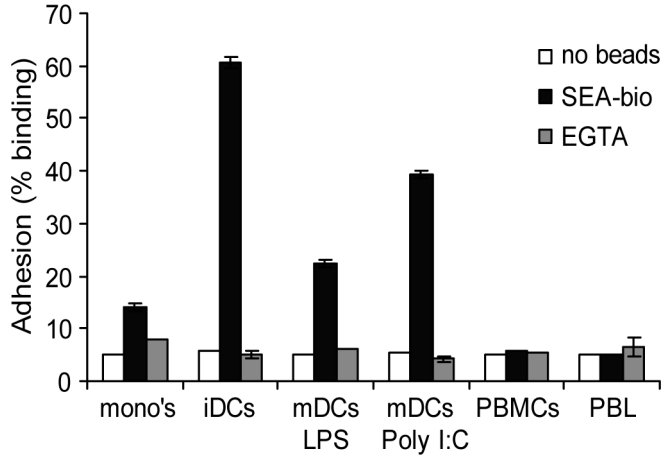

Figure 3. SEA-beads bind to immature and mature DCs. SEA was biotinylated and coupled to streptavidin-coated fluorescent beads (SEA-beads). Binding of the beads to cells was measured by FACScan analysis using Cellquest (Becton Dickinson, Oxnard, CA). SEA-beads show binding to immature and mature DCs. The binding of the beads was completely blocked by the calcium chelator EGTA.

Table 1A	iDCs	LPS	Poly I:C
DC-SIGN	1.26 ± 0.14	2.21% ± 1.96	15.60% ± 5.48
MGL	1.40 ± 0.26	0.39% ± 0.59	5.60% ± 8.55
MR	1.20 ± 0.09	0.49% ± 0.36	1.16% ± 0.72
DEC-205	0.11 ± 0.09	1175.20% ± 186.03	784.90% ± 261.14
DCIR	0.41 ± 0.04	4.62% ± 3.99	9.73% ± 5.27
Dectin-1	0.84 ± 0.14	2.94% ± 1.46	21.73% ± 1.75

Table 1B	iDCs	LPS	Poly I:C
TLR1	0.97 ± 0.21	30.85% ± 1.95	51.16% ± 1.01
TLR2	3.61 ± 0.24	3.96% ± 0.91	19.38% ± 1.79
TLR3	0.15 ± 0.07	175.00% ± 2.60	173.33% ± 9.53
TLR4	1.39 ± 0.12	27.42% ± 1.79	33.15% ± 8.24
TLR5	0.20 ± 0.05	96.25% ± 6.75	95.00% ± 5.01
TLR6	0.62 ± 0.09	6.11% ± 2.75	23.88% ± 4.54
TLR7	0.08 ± 0.07	1289.00% ± 8.50	2677.74% ± 3.58
TLR8	0.58 ± 0.27	107.29% ± 9.10	163.52% ± 9.63
TLR9	BD	BD	BD
TLR10	0.01 ± 0.01	775.00% ± 11.20	525.00% ± 3.30
CD14	0.56 ± 0.35	60.71% ± 5.13	69.91% ± 3.45
MD2	1.84 ± 0.22	26.59% ± 9.37	37.11% ± 10.62

Table 1C	DC-SIGN	MGL	MR
iDCs	1.26 ± 0.14	1.40 ± 0.26	1.20 ± 0.09
5 SEA	97.62% ± 1.68	95.01% ± 3.56	98.33% ± 2.96
50 SEA	94.44% ± 5.47	92.14% ± 2.07	99.16% ± 0.56
5 SEA + LPS	1.59% ± 2.62	0.43% ± 3.29	0.42% ± 2.55
50 SEA + LPS	1.58% ± 3.17	0.41% ± 3.57	0.39% ± 1.42
5 SEA + Poly I:C	17.15% ± 4.36	5.05% ± 4.71	1.33% ± 4.68
50 SEA + Poly I:C	17.94% ± 0.87	4.57% ± 5.28	1.27% ± 6.58

Table 1. Expression levels of TLRs and CLRs on immature DCs or DCs stimulated with LPS or poly I:C. (A and B) The relative abundance is shown for iDCs, which reflects the relative amount of target transcript with respect to the expression of the endogeneous reference gene GAPDH (including standard error). For LPS and poly I:C stimulated DCs expression is indicated as a percentage with standard error of the expression on iDCs. The primers used where tested on positive controls prior to use. BD, below detection limits. (C) The expression levels of DC-SIGN, MGL and MR are shown on iDC and DCs stimulated with LPS and poly I:C in the presence of SEA. The relative abundance is shown for iDCs. For the other conditions the percentage with standard error is given, similar as described in A and B. Data represent mean values out of 6 donors.

of Dectin-1 and DCIR (relative abundance of 0.84 and 0.41, respectively) and low levels of DEC-205 (Table 1A). Upon maturation with either LPS or poly I:C, down- or upregulation is indicated as a percentage of the expression on iDCs, which was set a 100%. Our data show that DC-SIGN is downregulated on mDCs to 2% (LPS) and 15 % (poly I:C) of the expression level of iDCs, and also MGL, MR, DCIR and Dectin-1 are downregulated on mDCs (Table 1A). In contrast, DEC-205 is upregulated on mDCs (1175 % and 784 %). We also analyzed the expression levels of TLRs, as these

are known to play a role in S. mansoni induced immune responses. Monocyte-derived DCs express all TLRs tested except TLR9. Upon maturation by LPS and poly I:C TLR1, TLR2, TLR4, TLR6, CD14 and MD2 are downregulated, whereas TLR3, TLR7 and TLR10 are upregulated. In DCs incubated with poly I:C, the TLR8 expression levels are upregulated. TLR5 expression levels remain unchanged upon maturation, as well as TLR8 in DCs stimulated with LPS (Table 1B). In table 1C we show that interaction of SEA with iDCs does not influence the expression levels of DC-SIGN, MGL and MR in the absence, nor in the presence, of the TLR ligands LPS or poly I:C. on either immature or mature DCs. Thus, DCs express a variety of receptors that could be involved in SEA recognition and in particular TLR-induced maturation regulates the expression level of these receptors.

C-type lectins interact with SEA

Next, the role of DC-expressed C-type lectins in the binding of SEA was analyzed. As is shown in figure 4A, iDCs express DCIR, DEC-205 and MR on the cell surface, in addition to DC-SIGN and MGL (filled histograms). On mature DCs, the expression of

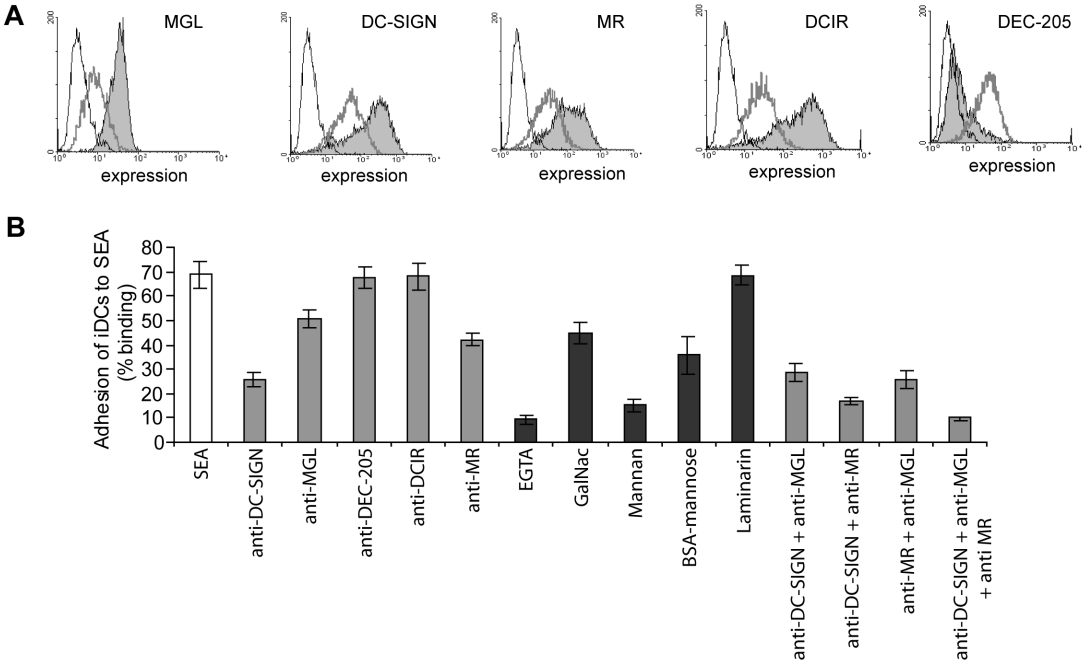

Figure 4. DCs bind SEA through the C-type lectins DC-SIGN, MGL and MR. (A) C-type lectin expression on immature and mature DCs. Open histograms represents the isotype control and filled histograms represent the anti-C-type lectin mAb staining on iDCs. The thick grey line shows C-type lectin expression on LPS matured DCs. The CLR amounts expressed on poly I:C matured DCs are comparable to those on LPS matured DCs (data not shown). (B) Immature DCs strongly bind SEA through DC-SIGN, MGL and MR. A bead adhesion assay was performed in the presence or absence of blocking mAbs directed against C-type lectins or the polysaccharide mannan (blocking DC-SIGN and MR), BSA-mannose (blocking the MR), laminarin (blocking Dectin-1), GalNAc (blocking MGL) or EGTA (blocking all CLRs). Experiments were performed in duplicates and one representative experiment out of three is shown.

DC-SIGN, MGL, MR and DCIR is reduced compared to iDCs, whereas higher amounts of DEC-205 protein are observed (Fig. 4A, thick grey lines), in agreement with mRNA levels (Table 1A). Specific antibodies to DC-SIGN and MGL could reduce binding of SEA to iDCs with 40%, indicating that DC-associated DC-SIGN and MGL bind to SEA (Fig. 4B). In addition, blocking antibodies to the mannose receptor (MR) could partially block binding, indicating that the MR is involved in the binding of SEA to iDCs. Addition of sugar inhibitors that are known to block interactions with these three CLRs (GalNAc (MGL), BSA-mannose (MR) and mannan (DC-SIGN/MR)), resulted in a comparable reduction in binding as found by using the anti-CLR antibodies as inhibitors. Remarkably, the binding of SEA to iDCs could be completely inhibited by a combination of antibodies against DC-SIGN, MGL and MR (Fig. 4B). By contrast, we could not observe inhibition of DC-binding to SEA using blocking antibodies against DEC-205[28] or DCIR (Fig. 4B), suggesting that these CLRs are not involved in binding. The lack of inhibition by laminarin, which inhibits binding to Dectin-1, suggests that Dectin-1 is not involved in binding of SEA to iDCs (Fig. 4B). Together these data indicate that iDCs recognize SEA through the CLRs DC-SIGN, MGL and the MR. The reduction of SEA binding to mature DCs (Fig. 3) corresponds to the observed lower expression of DC-SIGN, MGL and MR on mature DCs compared to immature DCs (Table 1A).

SEA is internalized by DCs and targeted to the MHC class II$^+$ LAMP$^+$ compartments

C-type lectins function as endocytic receptors on DCs and are either constitutively internalized from the cell surface, like the MR or internalized upon ligand binding, as we have previously shown for DC-SIGN[27]. We investigated whether SEA, upon binding by C-type lectins, is internalized from the cell surface. Indeed, we found that 50% of the SEA is internalized from the cell surface within 15-30 min (Fig. 5A). Next, we investigated which CLRs on iDCs facilitate this rapid internalization of SEA. Our results show that pre-incubation of DCs with blocking antibodies against DC-SIGN, MR or MGL, could partially block the internalization of SEA up to 25%, 30% and 35% respectively (Fig. 5B). However, the combination of antibodies against all three CLRs could block internalization of SEA completely, confirming that DC-SIGN, MGL as well as the MR are all involved in capture and subsequent internalization of SEA into DCs. In addition, colocalization of SEA with all three CLRs could be observed, using confocal laser scanning microscopy (CLSM) (Fig. 5C). The subcellular localization of SEA upon internalization was examined by staining with the lysosomal marker LAMP-1 or early endosomal marker EEA-1 (Fig. 5D). After 30 min, SEA was localized in EEA-1 positive compartments, whereas after 2 h all SEA was localized within LAMP-1 positive compartments, indicating that SEA captured by DC-SIGN, MGL and MR traveled within 2 h through endosomal compartments into lysosomes. At these later time points, SEA co-localized with MHC class II molecules (Fig 5E). Thus, we conclude that upon capture, SEA is rapidly internalized by DCs through the CLRs DC-SIGN, MGL and MR and targeted to the MHC class II$^+$ lysosomes, suggesting that SEA might be processed and presented.

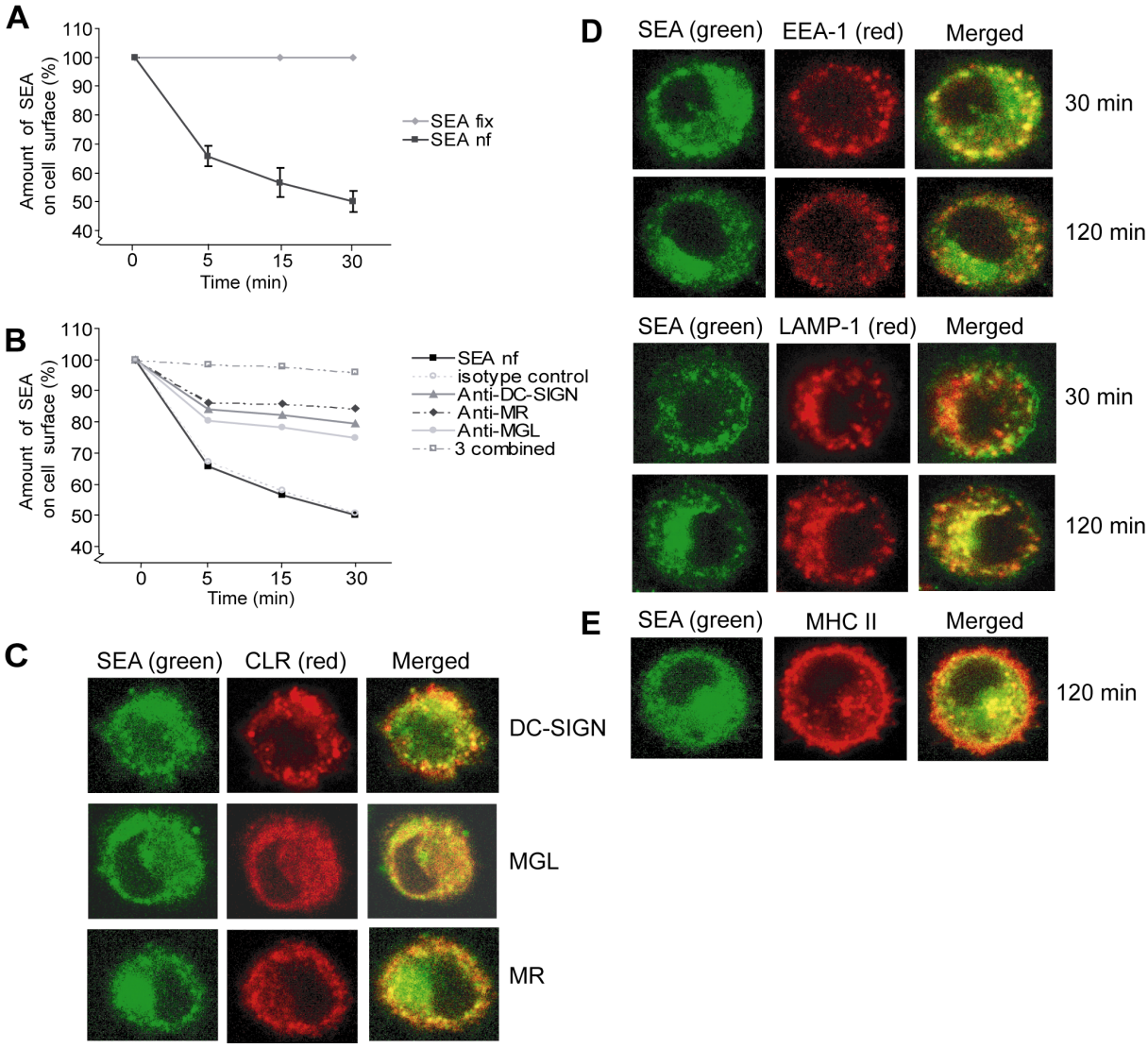

Figure 5. SEA is rapidly internalized by immature DCs. (A) Internalization of the cell surface bound biotinylated-SEA was analyzed on immature DCs using flow cytometry. Fixed cells were used to correct for the off rate of SEA at 37°C. Values represent mean of duplicates. One representative experiment out of three is shown. (B) Internalization of SEA from the cell surface of iDCs in the presence of mAbs directed against DC-SIGN, MGL, MR or combination of the three anti-CLR mAbs, or an isotype control mAb, was analyzed as in A. (C) Colocalization of SEA (green) with CLRs DC-SIGN, MGL and mannose receptor (red). (D) SEA (green) is targeted via early endosomes to the lysosomal compartment. DCs were incubated for 30 and 120 min with SEA. After fixation and permeabilization, cells were stained for LAMP-1 (lysosomes) or EEA-1 (early endosomes) (Red). (E) SEA (green) co-localizes with MHC II (red). After 120 min, there is colocalization inside the cell of internalized SEA and MHC II.

DISCUSSION

In schistosomiasis, eggs released by the adult worms are entrapped in host tissues and secrete soluble egg antigens (SEA), which results in the induction of a Th2 response[36-40]. The mechanism by which DCs induce SEA-specific Th2 responses during infection is not clearly understood. We show here that human monocyte-

derived iDCs pulsed with SEA, do not undergo a conventional maturation process *in vitro*. However, SEA is rapidly internalized through multiple CLRs and targeted into the MHC II$^+$ lysosomal compartments. Furthermore, SEA can inhibit the LPS- or poly I:C-induced DC activation and subsequent production of IL-12, TNFα, IL-6 and IL-10, resulting in modulation of the T cell polarizing capacity of DCs. From these data we hypothesize that internalization of SEA through CLRs on DCs leads to processing and presentation of SEA to T cells and may contribute to the outcome of the Th1/Th2 balance by providing a Th2 polarizing signal to the DCs. In addition, we propose that these CLRs may play a role in the SEA-induced downmodulation of LPS- or poly I:C-induced maturation and subsequent cytokine production.

Our data show that the C-type lectins DC-SIGN, MGL and the MR contribute to the internalization and targeting of SEA to MHC II$^+$ lysosomal compartments, where SEA can be processed and subsequently presented to T cells leading to a specific immune response. Lectin-mediated uptake pathways have been shown to be highly efficient to elicit immune responses[27,41].

Previously, we showed that DC-SIGN binds to SEA through the glycan antigens Galβ1-4(Fucα1-3)GlcNAc (Le^X) and GalNAcβ1-4(Fucα1-3)GlcNAc (LDNF)[8], whereas MGL recognizes both LDNF and GalNAcβ1-4GlcNAc (LDN) moieties within SEA[9]. The actual glycan ligands for the MR within SEA have not been identified yet. The MR preferentially targets mannose containing structures and differs from DC-SIGN as it does not interact with Le^X, despite having affinity for fucose[42]. At this point it remains unclear whether all schistosome antigens that are captured and internalized by DCs will be presented to T cells, or that there is a more selective form of presentation in which CLRs determine which antigens are presented and which are not.

The immunoregulatory role of DCs is believed to be determined by ligation of pathogen recognition receptors such as TLRs and CLRs, and signaling pathways induced by these receptors, which can interconnect through a so-called crosstalk. Interestingly, a recent study shows that targeting the MR could prime a regulatory program in DCs, leading to the production of anti-inflammatory cytokines and induction of Th2 cells with regulatory capacity[43]. In addition, the production of inflammatory cytokines by the TLR4-ligand LPS was prevented implying crosstalk between the MR and TLR4. Several examples illustrate that pathogen-induced signaling through DC-SIGN results in a shift in Th1/Th2 balance. Signaling through DC-SIGN by Lewis positive *Helicobacter pylori* results in a shift in the Th1/Th2 balance towards Th2, whereas Lewis negative *H. pylori* do not bind DC-SIGN and trigger Th1 responses[19]. Lactobacillus species *L. reuteri* and *L. casei* instruct DCs to induce IL-10 producing regulatory T cells that suppress T cell responses through engagement of DC-SIGN[35]. *Mycobacterium tuberculosis* interacts with DC-SIGN on DCs through its cell wall component ManLAM, which inhibits the TLR-induced maturation and induces the immunosuppressive cytokine IL-10[21]. *Neisseria meningitidis* expressing *IgtB* LPS targets DC-SIGN and skewed T cell responses driven by DCs towards Th1 activity[22]. These reports suggest that CLRs could play an important role in

determining the outcome of the Th1/Th2 balance.
More insight in the signaling capacities of DC-SIGN was recently provided by studies of Caparros *et al*[44], which showed that ligation of DC-SIGN results in the phosphorylation of ERK1/2 and Akt. Interestingly, DC-SIGN ligation synergizes with TNFα receptor-initiated signals for enhanced IL-10 release. Our observation that copulsing of DCs with SEA and LPS inhibits the capacity of LPS to activate DCs to produce IL-10, despite the binding of SEA to DC-SIGN, indicates that SEA in addition triggers other receptors that participate in the integration of the intracellular signals to generate the SEA specific immune responses. Such receptors may include the MR and/or MGL, but other unidentified TLRs or CLRs cannot be excluded.
Our data show that DCs, pulsed with SEA, do not mature and cannot induce a T cell response *in vitro*. In addition, coculture of SEA with cell lines transfected with TLR2 or TLR4, respectively, did not lead to production of IL-8 (data not shown). Although these data do not exclude the presence of TLR ligands in the SEA preparation, their concentrations apparently are too low to induce maturation signals that support the differentiation of naive T cells *in vitro*. However, *in vivo* TLR agonists may be provided via other components of the eggs or worms. Aksoy and coworkers showed that living *S. mansoni* eggs activate murine bone marrow-derived DCs through TLR2 and TLR3 engagement[10]. Egg-derived RNA possessed RNase A-resistant and RNase III-sensitive structures, suggesting the presence of double-stranded structures, which are capable of triggering TLR3 activation. In addition, the schistosome-specific lysophosphatidylserine was shown to activate human monocyte-derived DCs through TLR2, which results in the ability of DCs to induce the development of IL-10 producing regulatory T cells as well as the induction of a Th2 response[12]. Thus, since TLR2, TLR3 and TLR4 ligands have been implicated in schistosomiasis[10-12], our data showing that SEA inhibits the poly I:C (TLR3) and LPS (TLR2/TLR4)-induced maturation may have *in vivo* functional relevance.
Experimental infection of mice with *S. mansoni* has provided an extensively used model for examining the development and role of Th2 responses driven by egg antigens. Whereas SEA in the human system similarly as in mice has a Th2 polarizing capacity, some differences in the mechanism are observed between the murine and human system[36-40]. Kane and coworkers reported that in BM-DCs of C57BL/6 mice SEA is able to downmodulate LPS induced maturation, IL-12 production and to upregulate IL-10 production[39]. By contrast, we observed downregulation of both IL-12 and IL-10 using human monocyte-derived DCs upon copulsing with SEA and LPS, or poly I:C, respectively. In addition, whereas we observed a rapid internalization and targeting of SEA to MHC II positive lysosomal compartments by human DCs, Cervi *et al* reported that murine bone marrow-derived DCs internalized SEA, but the SEA stayed in LAMP-2 negative compartments unless a maturation stimulus was supplied[37]. The observed differences between the mouse and human system may reflect differences in DC receptors that are triggered by SEA. In particular the expression pattern and specificity of C-type lectins may be different between the mouse and human system.

Thomas *et al* showed that HSA-conjugated Lacto-*N*-fucopentaose III, which contains Le^X, can activate murine bone marrow-derived DCs into a Th2 inducing phenotype through a TLR4-dependent mechanism[11]. LNFP III–TLR4 interactions in mice induce signaling through the ERK pathway, and differs from the LPS-induced TLR4 activation that leads to intracellular signaling through three MAP-kinase signaling pathways: ERK, p38 and JNK[11]. Remarkably, signaling through DC-SIGN also leads to ERK signaling without p38 stimulation, and thus resembles the LNFP III-TLR4 signaling in that respect. Although many receptors will participate in the integration of the intracellular signals to generate SEA-specific responses in schistosomiasis, it is tempting to speculate that the Le^X-DC-SIGN interaction in humans has a similar function as the Le^X–TLR4 interaction in the murine system, leading to ERK-signaling and contributing to a Th2-inducing DC maturation. It would be interesting to investigate whether bone marrow-derived murine DCs show next to TLR4-Le^X interaction, also a (C-type) lectin-mediated binding of Le^X or other carbohydrate determinants. Clearly, the dissection of the receptors that interact with Schistosome egg antigens, as well as the intracellular signals triggered upon binding, need to be further investigated both in the human and murine system to clarify the role of the different pathogen recognition receptors in the generation of the egg-specific Th2 responses.

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CHAPTER 10

NEISSERIA MENINGITIDIS EXPRESSING *lgtB* LIPOPOLYSACCHARIDE TARGETS DC-SIGN AND MODULATES DENDRITIC CELL FUNCTION

Liana Steeghs, Sandra J. van Vliet, Heli Uronen-Hansson, Andries van Mourik, Anneke Engering, Marta Sanchez-Hernandez, Nigel Klein, Robin Callard, Jos P.M. van Putten, Peter van der Ley, Yvette van Kooyk and Jan G.J. van de Winkel

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ABSTRACT

Neisseria meningitidis lipopolysaccharide (LPS) has been identified as a major determinant of dendritic cell (DC) function. Here we report that one of a series of meningococcal mutants with defined truncations in the lacto-*N*-neotetraose outer core of the LPS exhibited unique strong adhesion and internalization properties towards DCs. These properties were mediated by interaction of the GlcNAc β1-3Galβ1-4Glc-R oligosaccharide outer core of *lgtB* LPS with the dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) lectin receptor. Activation of DC-SIGN with this novel oligosaccharide ligand skewed T cell responses driven by DCs towards T helper type 1 activity. Thus, the use of *lgtB* LPS may provide a powerful instrument to selectively induce the desired arm of the immune response and potentially increase vaccine efficacy.

INTRODUCTION

Dendritic cells (DCs) are crucial in the initiation of pathogen-specific immune responses, both during natural infection and in response to vaccination. The immunoregulatory role of DCs is believed to be determined by ligation of specific pattern recognition receptors (PRRs). This process results in the development of different DC subsets that induce differentiation of naive T cells into T helper 1 (Th1) cells or Th2 cells. The best studied PRR families on DCs are Toll-like receptors (TLRs) that recognize specific pathogen-derived components such as the lipid A moiety of lipopolysaccharide (LPS), and C-type lectin receptors (CLRs) that are specific for pathogen-derived carbohydrate structures. Triggering of TLRs leads to the production of regulatory cytokines and upregulation of MHC and costimulatory molecules required for T cell signaling[1], while ligation of C-type lectins has been demonstrated to result in binding and internalization of pathogens for antigen processing and presentation to T cells[2].

One bacterial structure that appears to have the ability to act both as a potent adjuvant[3] and elicitor of a specific immune response[4,5], is *Neisseria meningitidis* LPS. We recently demonstrated that a completely LPS-deficient *N. meningitidis* mutant is very poorly internalized by DCs compared to the parent strain and lacks the capacity to fully activate DCs[6]. This mutant carries a defect in lipid A biosynthesis and therefore lacks both the lipid A and carbohydrate moiety of the LPS. The molecular basis for the altered interaction with DCs thus remains to be defined. Here we addressed this question and deciphered the possible modulatory effects of the variable lacto-*N*-neotetraose oliogosaccharide core of *N. meningitidis* LPS on DC function using a defined panel of oligosaccharide outer core mutants which has previously been described[7,8]. Phagocytosis and receptor binding studies, demonstrated that DCs much more efficiently phagocytosed the *lgtB* mutant than the parent strain and that this interaction was uniquely mediated via the DC-SIGN C-type lectin receptor. Furthermore, T cell responses driven by DCs pulsed with the

lgtB mutant showed a marked shift towards the Th1 function. These data suggest that the oligosaccharide structure of meningococcal LPS is a major determinant of DC function and that the *lgtB* oligosaccharide may be a powerful new tool to specifically target DC-SIGN generating a beneficial Th1 response and increased vaccine efficacy.

MATERIALS AND METHODS

Bacterial strains

The oligosaccharide mutants (structures shown in fig. 1) were derived from the wildtype group B *N. meningitidis* H44/76 and have been described previously[7-9]. No phenotypical differences were apparent for the wildtype and the oligosaccharide mutants with respect to pili, capsular polysaccharide and major outer membrane proteins. The H44/76 *opa*-deficient mutant was constructed by insertional deletion of all four *opa* genes (Netherlands Vaccine Institute, Peter van der Ley). *N. meningitidis* strain M992 and *N. gonorrhoeae strain F62* and its *lgtB* mutant, generously provided by E.C. Gotschlich (Rockefeller University, New York, USA), have been described previously[10,11]. Strains were grown on gonococcal agar (Difco, Basingstoke, UK) supplemented with Vitox (Oxoid, Basingstoke, UK) in an atmosphere of 5% CO_2 at 37°C for 18 h. Bacteria were inactivated in 0.5% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15 min and washed thoroughly in RPMI 1640 medium without phenol red (Gibco, Paisley, UK). FITC-labeled bacteria were prepared by incubation with 0.5 mg/ml of FITC (Sigma, Poole, UK) for 20 min at 37°C followed by extensive washing. Bacterial suspensions with an optical density at 540 nm of 1, corresponding to 10^9 bacteria/ml, were prepared in RPMI 1640 medium without phenol red. All bacteria were equally FITC-labeled as determined by FACS.

Cell culture

DCs were generated from human peripheral blood mononuclear cells (PBMCs) as described previously[12,13]. Monocytes were prepared from PBMCs by centrifugation over Percoll and incubated for 7 days in RPMI supplemented with 10% heat inactivated FCS, 2.4 mM L-glutamine, 100 U/ml Penicillin-streptomycin (all from Gibco), 100 ng/ml of human recombinant GM-CSF and 50 ng/ml of human recombinant IL-4 (both from Schering-Plough, Welwyn Garden City, Herts UK). Immature DCs, prepared in this way were $CD14^{low}$, $CD83^{-}$, $CD86^{low}$, $CD25^{-}$, expressed HLA-DR, HLA-DQ, HLA Class I, CD40, CD11c and CD1a, and were negative for both CD19 and CD3.

HEK293T cells were maintained in DMEM (Gibco), supplemented with 10 mM HEPES, non-essential amino acids, sodium pyruvate and 10% FCS. HEK293T were transfected using lipofectamine (Gibco) according to the manufacturer's protocol. Briefly, 10 μg of plasmid DNA was co-incubated with 54 μl of lipofectamine in a volume of 1.2 ml Optimem (Gibco) for 30 min at room temperature. Cells were washed once with Optimem, after which the DNA/lipofectamine mixture was added

to the cells. After 6 h at 37°C, medium was replaced for normal culture medium. At day 2 after transfection, cells were harvested and expression of C-type lectin receptors was tested using specific monoclonal antibodies. Binding of FITC-labeled bacteria to transfected HEK293T cells was analyzed on a FACSCalibur after incubation of cells and bacteria at a ratio of 1:50 for 45 min at 37°C.

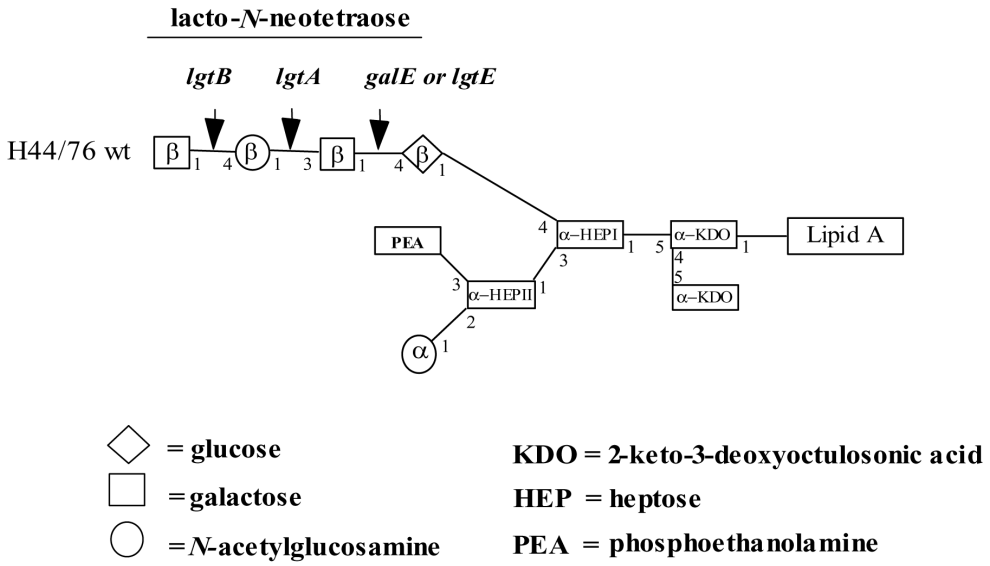

Figure 1. Schematic representation of *N. meningitidis* H44/76 wild type L3 LPS. Stepwise-truncated oligosaccharide mutants were generated by insertional inactivation of genes encoding glycosyltransferases (indicated in italic)[7,8]. The Arabic numbers mark positions of the binding sites; α and β indicate the anomeric configuration.

DC binding and uptake

DCs at a concentration of 5 x 10^5/ml were incubated with FITC-labeled bacteria at DC/bacteria ratio of 1:100 in RPMI 1640 supplemented with 10% FCS for periods of time between 30 min and 24 h. DCs were fixed in 2% paraformaldehyde (PFA), washed and analyzed on a FACSCalibur. DCs associated with bacteria were easily identified by fluorescence within the DC gated population. For confocal microscopy, DCs stimulated with FITC-labeled bacteria were allowed to adhere for 10 min to an adhesion slide (Biorad, Herts, UK) and subsequently fixed with 4% PFA for 10 min. DCs were visualized by incubation of 5 μg/ml of anti-MHC Class II mAb for 1 h (Dako, Glostrup, Denmark), followed by detection with 5 μg/ml of Texas Red-conjugated goat anti-mouse antibody (Molecular probes) for 1 h. The slides were washed and mounted in Citifluor (Citifluor, UK). Confocal images were obtained using a Leica SP2 confocal laser scanning microscope system (Leica, Milton Keynes, UK) fitted with appropriate filter sets. To identify intracellular bacteria, 15-20 optical sections (0.2-0.5 μm) spanning the entire DC were projected and superimposed with Leica confocal imaging software. The binding specificity of the *lgtB* mutant to DCs was determined by incubating immature DCs with either 5 mM EGTA or 20 μg/ml anti-DC-SIGN monoclonal antibody AZN-D1 for 30 min at 37°C prior to incubation

with bacteria.
For all DC experiments, representative data from 3 independent experiments are shown. Data were not combined since a donor-dependent variation in the strength of association was observed with variations in the maximum of positive cells in the gated population of 25-70% and 70-100 % for strain H44/76 and the *lgtB* mutant, respectively.

Cytokine measurements
Cytokines were measured by ELISA with CytoSets™ ELISA kits (Biosource, Nivelles, Belgium) for human IL-12 and IL-10 according to the manufacturer's instructions. The results were analyzed by paired t-test. P-values <0.05 were considered to be statistical significant.

Soluble C-type lectin-Fc adhesion assay
C-type lectin-Fc molecules consist of the extracellular portion of the C-type lectin receptor fused at the COOH terminus to a human IgG1 Fc fragment. In case of DC-SIGN, MGL, Dectin-1B and DCIR, this extracellular portion corresponds to amino acid residues 64-404, 61-289, 69-201 and 70-237, respectively. The soluble C-type lectin-Fc adhesion assay was performed as follows. *N. meningitidis* whole cells (OD540=0.1) were coated onto ELISA plates (100 µl/well) in phosphate buffered saline (PBS) for 18 h at room temperature (RT), followed by blocking with 1% bovine serum albumine (BSA) for 30 min at 37°C. Soluble C-type lectin-Fc supernatant was added for 2 h at RT. Unbound C-type lectin-Fc was washed away and binding was determined by anti-IgG1 Fc ELISA.

DC-driven Th1/Th2 differentiation
Immature DCs (CD11c-positive) were cultured from monocytes of healthy donors in Iscove's Modified Dulbecco's Medium (Gibco), supplemented with 10% FCS (BioWithaker, Verviers, Belgium), 500 U/ml IL-4 and 800 U/ml GM-CSF (both from Schering-Plough)[12]. At day 6, DC maturation was induced with PFA-fixed *N. meningitidis* wildtype and *lgtB* mutant at a multiplicity of infection of 10. The following positive controls were included in the assay (i) mixed Th1/Th2 response, 10 ng/ml *Escherichia coli* LPS (Sigma-Aldrich, St. Louis, MO) (ii) Th1 differentiation, 20 µg/ml poly I:C (Sigma-Aldrich) and (iii) Th2 differentiation, 10 µg/ml PGE2 and 10 ng/ml LPS (Sigma-Aldrich). At day 2, DCs were washed and incubated with heterologous $CD45RA^+/CD4^+$ T cells (20×10^3 T cells/5×10^3 DCs). In parallel, DCs were analyzed for maturation markers (CD80, CD83, CD86 and HLA-DR) by flow cytometry. At day 12-15, quiescent T cells were restimulated with 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) for 6 h. After 1 hour 10 µg/ml Brefeldin A (Sigma-Aldrich) was added to the T cells. Single cell production of IL-4 and IFNγ was determined by intracellular flow cytometric analysis. Cells were fixed in 2% PFA, permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with anti-human IFNγ-FITC and anti-human IL-4-PE (BD Pharmingen, San Diego, CA).

RESULTS

Enhanced DC binding and internalization of a N. meningitidis lgtB mutant critically depends on the expression of the GlcNAcβ1-3Galβ1-4Glc-R oligosaccharide core.

To determine the influence of the lacto-*N*-neotetraose oligosaccharide outer core of *N. meningitidis* LPS on DC binding and internalization, immature DCs were co-cultured with PFA-fixed FITC-labeled *N. meningitidis* strain H44/76 and its oligosaccharide mutant derivatives *lgtB*, *lgtA* and *galE* (Fig. 1). Time-dependent increases in DC association were observed for both PFA-fixed wildtype, and mutant bacteria (Fig. 2A and 2B). Notably, a significantly enhanced DC association was observed for the *lgtB* mutant, as compared to the wildtype and the other oligosaccharide mutants. Consistently, greatly enhanced internalization was seen for the *lgtB* mutant by confocal microscopy (Fig. 2C). DC association comparable to wildtype was observed for an H44/76 *lst* mutant, incapable of sialylating its L3 LPS, demonstrating that the lower DC association of the wildtype as compared to *lgtB* cannot be caused by sialylation of wildtype LPS (Fig. 2D). Furthermore, a role for the variable neisserial Opa adhesins was excluded by the similar DC binding of a genetically defined Opa-deficient derivative of H44/76, carrying deletions in all four *opa* genes (Fig. 2D).

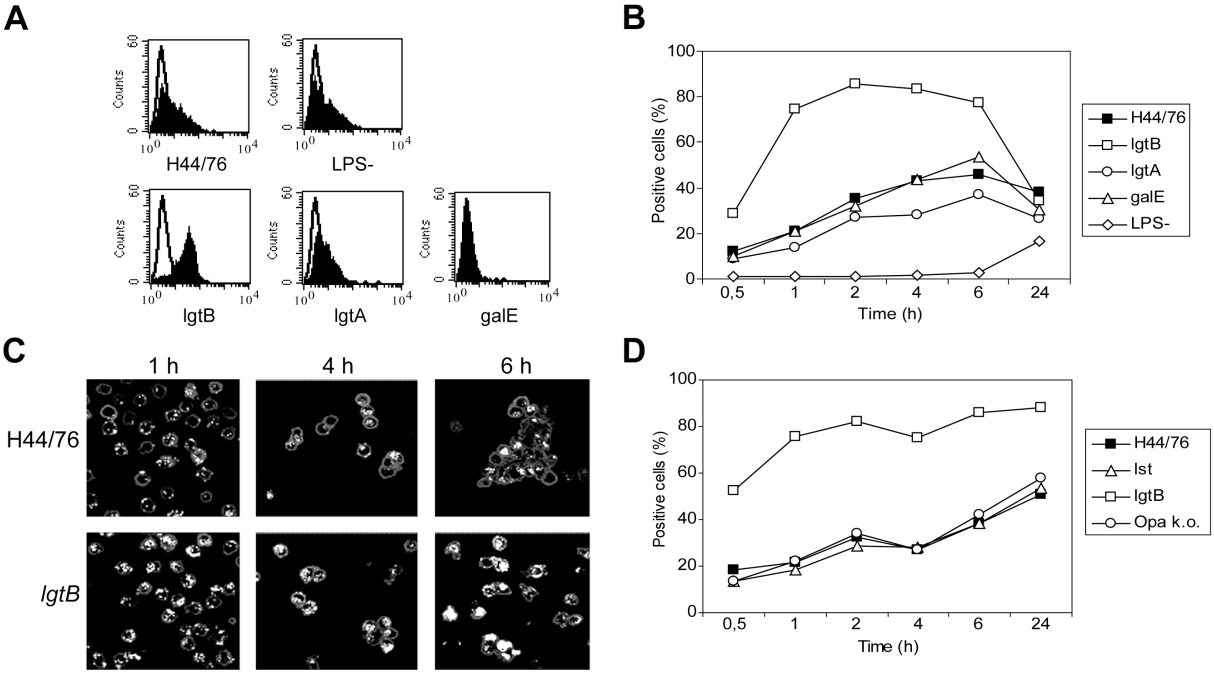

Figure 2. Interaction of *N. meningitidis* wildtype H44/76 and its oligosaccharide and *opa-deficient* derivatives. Immature DCs were co-cultured with PFA-fixed FITC-labeled bacteria. At the indicated time points samples were taken to measure association or internalization of bacteria by DCs. (A) FACS plot of bacterial-DC association at 2 h. (B and D) Time course of bacterial-DC association. The reduced number of positive DCs at 24 h is presumably caused by loss or quenching of the FITC-signal of processed bacteria (C) Internalization of bacteria as viewed by confocal microscopy. Representative data of three separate experiments using DCs from three different donors are shown.

To demonstrate that the GlcNAcβ1-3Galβ1-4Glc-R outer core structure as present on the *lgtB* mutant LPS is essential for the enhanced binding and uptake, DC-association was investigated for *N. meningitidis* strain M992. This strain naturally carries an LPS of immunotype L6 that has the same terminal GlcNAcβ1-3Galβ1-4Glc-R outer core as *lgtB* LPS. Greatly enhanced DC association comparable to the *lgtB* mutant was seen for M992 (Fig. 3). Similar enhanced DC binding was found for a *Neisseria gonorrhoeae lgtB* mutant as compared to its wildtype strain (data not shown). Taken together, these data demonstrate that the enhanced DC binding and internalization of the *N. meningitidis lgtB* mutant critically depends on the presence of the GlcNAcβ1-3Gal β1-4Glc-R LPS outer core.

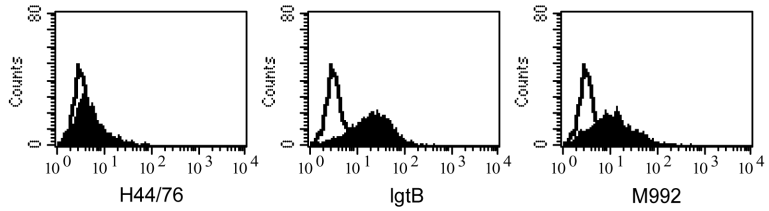

Figure 3. DC association of *N. meningitidis* strain M992. Immature DCs were cultured in the presence of PFA-fixed FITC-labeled bacteria of *N. meningitidis* wildtype strain H44/76, its *lgtB* mutant, and immunotype L6 strain M992. After 2 h, bacterial-DC association was assessed by FACS analysis. Depicted FACS plots represent typical data of three separate experiments using DCs from three different donors.

Binding/uptake of the lgtB mutant is mediated via DC-SIGN

The difference in association and uptake of the wildtype and the *lgtB* mutant prompted us to investigate whether this process was mediated via CLRs that are abundantly expressed on immature DCs. Binding of PFA-fixed wildtype and the *lgtB* mutant to DCs was studied in the presence or absence of 5 mM of the Ca^{2+}-chelator EGTA, which inhibits the Ca^{2+}-dependent CLR function. In the presence of EGTA, DC association of both wild type and the *lgtB* mutant was completely abrogated, consistent with a role of members of the CLR family (data not shown). To identify C-type lectins involved in the interaction we determined the binding of C-type lectin-Fc chimeras of dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN)[14], macrophage galactose-type lectin (MGL)[15], Dectin-1B[16] and dendritic cell immunoreceptor (DCIR)[17], to the parental strain, the oligosaccharide mutants, and the LPS-deficient mutant in a receptor-adhesion assay (Fig. 4A). Whereas the wildtype, *lgtA*, *galE* and LPS-deficient mutant did not bind any of the C-type lectin-Fc chimeras, strong binding of DC-SIGN-Fc was found for the *lgtB* mutant. Consistently, the *lgtB* mutant adhered to DC-SIGN-transfected HEK293T cells, but not to MGL, Dectin-1B, DCIR, or mock-transfected HEK293T cells (Fig. 4B). To determine the specificity of *lgtB* LPS for DC-SIGN the association of the *lgtB* mutant with DCs was assessed in the presence or absence of the blocking anti-DC-SIGN antibody AZN-D1 (Fig. 4C). In the presence of AZN-D1, binding of *lgtB* by DCs was

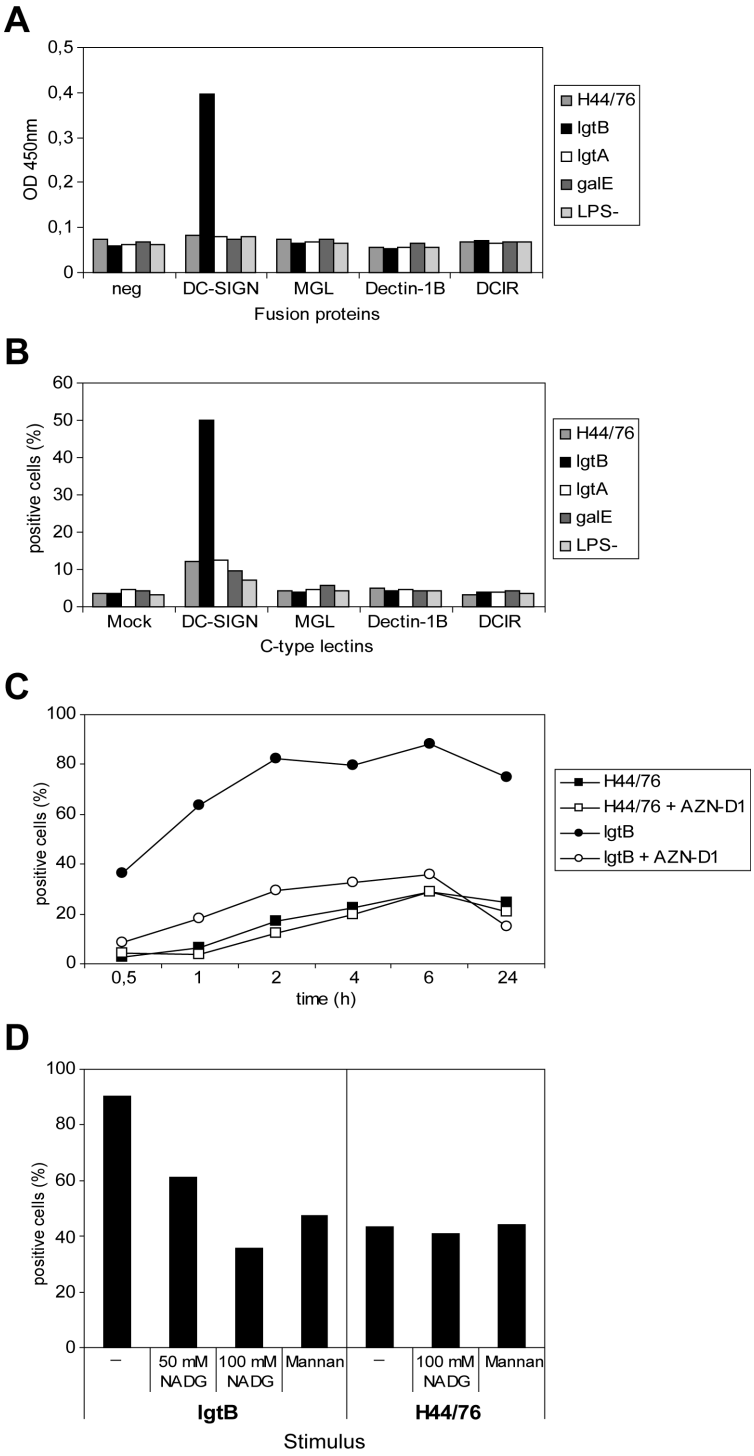

Figure 4. Interaction of *N. meningitidis* wildtype H44/76 and its oligosaccharide mutants with C-type lectins. (A) Binding of *N. meningitidis* wildtype and oligosaccharide mutants to C-type lectin Fc chimeras in a soluble adhesion assay. (B) Binding of *N. meningitidis* wildtype and oligosaccharide mutants to C-type lectins transiently transfected in HEK293T cells. (C) Effect of DC-SIGN-blocking antibody AZN-D1 on binding of *N. meningitidis lgtB* mutant to immature DCs. (D) Effect of GlcNAc and yeast mannan on binding of *N. meningitidis lgtB* mutant to immature DCs after 30 min of co-culture. Representative data of three separate experiments are shown.

reduced to wildtype levels, clearly demonstrating that DC-SIGN is critically involved in the binding and uptake of the *lgtB* mutant by immature DCs.

To assess whether the terminal GlcNAc of the *lgtB* oligosaccharide core is involved in binding to DC-SIGN, we next determined DC association of the *lgtB* mutant and the wildtype in the presence of N-acetyl-D-glucosamine (NADG) and, as a control, yeast mannan (Fig. 4D) which has previously been demonstrated to block binding to DC-SIGN[14]. As expected, yeast mannan, but also NADG inhibited association of the *lgtB* mutant to DCs in a dose-dependent manner. Under identical conditions the wildtype

strain was hardly impaired in DC association in the presence of NADG and mannan. Together these results indicate that the terminal GlcNAc of the *lgtB* oligosaccharide core plays a crucial role in the binding to DC-SIGN.

DC association of live lgtB mutant is mediated via DC-SIGN

We next determined whether DC binding/internalization of *N. meningitidis lgtB* mutant via DC-SIGN was also observed for live bacteria (Fig. 5). DC association was studied with live FITC-labeled wild type and oligosaccharide mutant bacteria in the presence or absence of AZN-D1 blocking antibody. Viable *lgtB* mutant bacteria associated efficiently with DCs, while hardly any DC association was found for viable wildtype as well as *lgtA* and *galE* mutant bacteria. In fact, the association of these strains was even much lower than observed for their inactivated counterparts (Fig. 2B). Binding of the *lgtB* mutant was inhibited in the presence of the DC-SIGN-specific blocking antibody AZN-D1, demonstrating that DC-SIGN also mediates DC association of live *N. meningitidis lgtB* mutant.

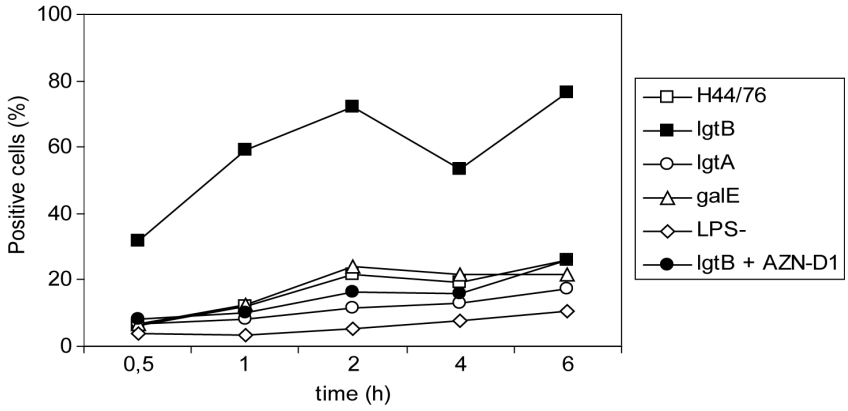

Figure 5. DC association of live *N. meningitidis* wildtype H44/76 and its oligosaccharide mutants. Immature DCs were cultured with FITC-labeled bacteria in the presence or absence of the anti-DC-SIGN blocking antibody AZN-D1. At the indicated times, samples were taken to measure DC association of bacteria. Representative data of three separate experiments are shown.

Targeting of the lgtB mutant to DC-SIGN initiates a Th1 immune response

To assess the functional relevance, if any, of the interaction of *lgtB* mutant LPS with DC-SIGN, we studied DC maturation, cytokine production, and DC driven T cell responses after pulsing immature DCs with either PFA-fixed wildtype or mutant bacteria. No differences were found in maturation of DCs pulsed with wildtype or mutant bacteria as assessed by expression of costimulatory molecules and maturation markers (data not shown). Similarly, the enhanced internalization of the *lgtB* mutant as compared to the wildtype was not accompanied by an alteration in the induction of the effector cytokines IL-10 and IL-12p70 (Fig. 6A; IL-10 (3h) p=0.1, IL-10 (18h), p=0.5, IL-12 (18h) p=0.4).

To assess the DC driven T cell responses, the ability of DCs pulsed with PFA-fixed wildtype or *lgtB* mutant to induce Th1 or Th2-profiles, as deduced from measured IL-4 and IFNγ production in T cells, was studied. To monitor donor heterogeneity in

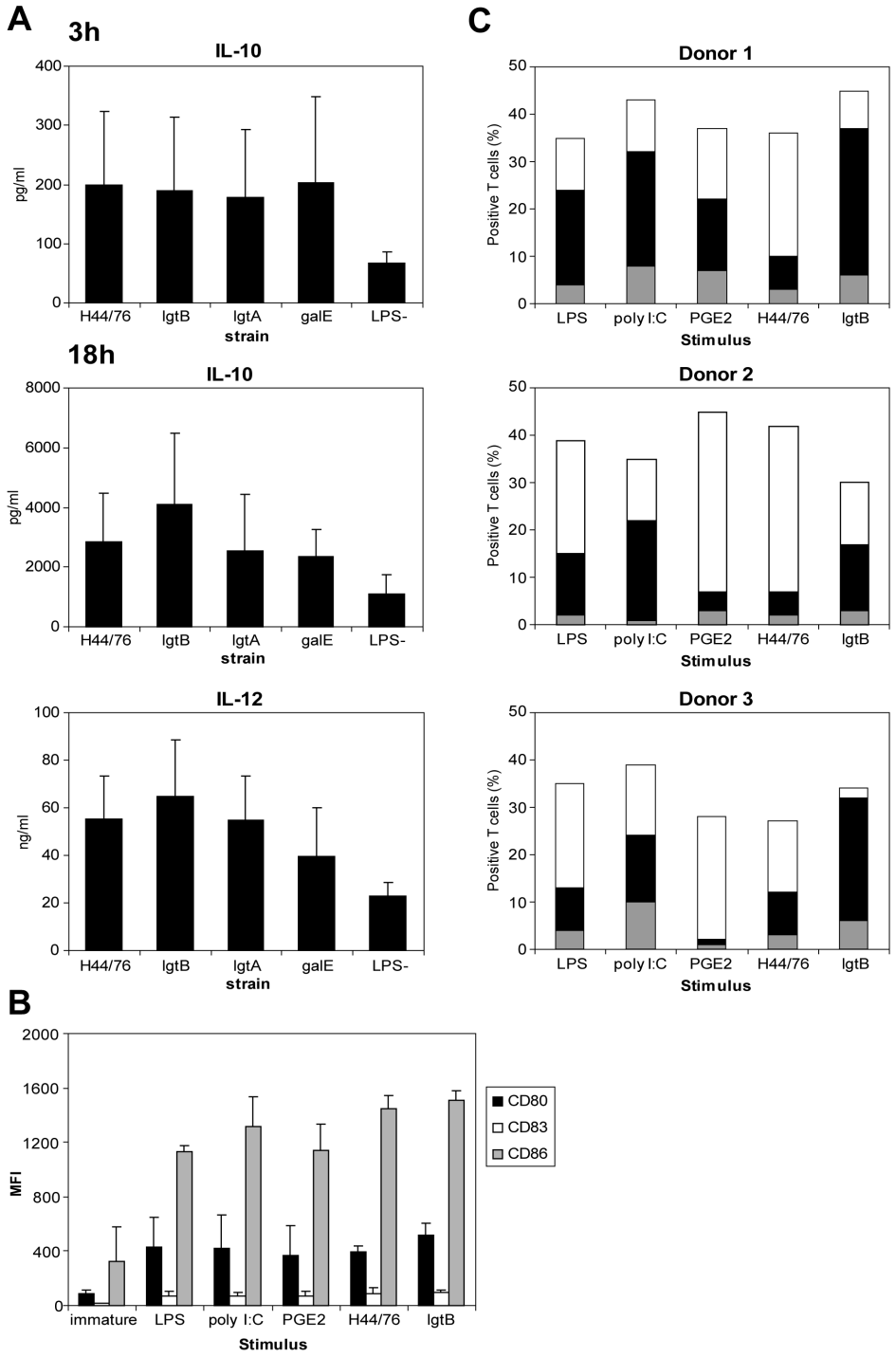

Figure 6. Functional assessment of DC activation in response to *N. meningitidis* wildtype and its oligosaccharide mutants. (A) Cytokine production by immature DCs after 3 h (IL-10) and 18 h (IL-10 and IL-12) of coculture of DCs with PFA-fixed FITC-labeled *N. meningitidis* wildtype H44/76 and its oligosaccharide mutants as measured by ELISA. IL-12 levels at 3 h of infection were below levels of detection. Cytokine measurements at 3 h and 18 h are from different donor pairs. (B-C) DCs were incubated with PFA-fixed *N. meningitidis* wildtype H44/76 and its *lgtB* mutant, *E. coli* LPS, poly I:C or PGE2 for 48 h, washed and (B) analyzed for maturation markers (mean and SEM of three separate experiments) or (C) cocultured with highly purified $CD45RA^+CD4^+$ T cells. Quiescent T cells were restimulated with PMA and ionomycin, and intracellular IL-4 (white), IFNγ (black) and IL-4/IFNγ (grey) was analyzed on a single cell basis by flow cytometry. Data from three different donors are shown.

Th1/Th2 profiles, *Escherichia coli* LPS, poly I:C and PGE2 were included as controls for a mixed Th1/Th2, Th1 and Th2 responses, respectively. In these experiments, again no differences in DC maturation as assessed by expression of costimulatory molecules CD80, CD83 and CD86, was found between the wildtype and the *lgtB* mutant bacteria (Fig. 6B). Assessment of the T cell response demonstrated that DCs pulsed with *N. meningitidis* wildtype predominantly generated a Th2 type immune response as the majority of T cells were producing IL-4, whereas DCs pulsed with the *lgtB* mutant predominantly evoked IFNγ-producing T cells (Fig. 6C). The apparent ability of the *lgtB* mutant to shift the Th1 versus Th2 cell balance towards Th1 was observed in all 3 donors, irrespective whether they were more prone towards Th1 (donor 1) or Th2 (donor 2 and 3) as can be deduced from the included controls**.** These data indicate that specific targeting of *lgtB* LPS towards DC-SIGN generates DC signals that drive the immune response into Th1.

DISCUSSION

DCs are highly specialized antigen presenting cells that form a gateway between the innate and adaptive immune system. Identification of molecules that modulate DC function paves the way to selectively induce the desired arm of the immune response. We evaluated a panel of *N. meningitidis* LPS oligosaccharide mutants for their DC activating potential. This led to the discovery that *N. meningitidis lgtB* LPS, expressing a GlcNAcβ1-3Galβ1-4Glc-R outer core targets the C-type lectin receptor DC-SIGN thereby mediating highly efficient bacterial uptake, and skewing of the immune response into a Th1 direction.

DC-SIGN has previously been demonstrated to bind high mannose oligosaccharides and fucose-containing Lewis blood group antigens[18,19]. The interaction of *lgtB* LPS with DC-SIGN apparently critically depends on the expression of the terminal *N*-acetylglucosamine (GlcNAc) residue, as both wildtype LPS and *lgtA* LPS (that lack a terminal GlcNAc), do not bind to this receptor. Consistently, DC association of the *lgtB* mutant was inhibited in the presence of GlcNAc emphasizing the importance of the GlcNAc residue in the interaction with DC-SIGN. In agreement with these findings is the observation by Klena *et al* (2005) that DC-SIGN ligation by *E. coli* might depend on the GlcNAc residue expressed in the oligosaccharide core of *E. coli* LPS[20]. However, due to the limited number of oligosaccharide mutants tested in this study and the lack of GlcNAc inhibition data, it cannot be ruled out that another sugar of the *E. coli* oligosaccharide core mediates the interaction with DC-SIGN. Indeed, it is interesting that a sugar monomer like GlcNAc can inhibit DC-SIGN ligation whereas other monomers, like mannose cannot. Still, glycan arrays previously used to determine DC-SIGN oligosaccharide specificity did not reveal GlcNAc as a DC-SIGN ligand[21]. It is therefore likely, that DC-SIGN specificity for GlcNAc critically depends on the oligosaccharide backbone on which it is presented. Moreover, it is notable, that co-crystal structures of the carbohydrate recognition domain (CRD) of DC-SIGN bound to a di-antennary pentasaccharide containing 3

mannose and 2 terminal GlcNAc residues, revealed that one of the terminal GlcNAc residues forms a crosslink by interacting with the principal Ca^{2+}-site of another CRD, thereby forming a dimer[19]. Thus, oligosaccharide antigens expressing GlcNAc as terminal sugars might represent a novel group of antigens that can bind DC-SIGN. In addition, the crosslinking capacity of GlcNAc might modulate receptor function differently than previously demonstrated for other ligands of DC-SIGN.

Although *lgtB* LPS is apparently the only meningococcal mutant LPS structure efficiently recognized by DC-SIGN, other LPS-binding surface receptors must exist, as binding of the LPS-deficient *lpxA* mutant to DCs is much reduced as compared to the wildtype[6] and the other oligosaccharide mutants (Fig. 2). Moreover, the existence of other receptor(s) that participate in uptake of the *lgtB* mutant cannot be excluded, since the DC-SIGN-blocking antibody does not completely abrogate DC association but reduces it to the wildtype level. In contrast to the *lgtB* LPS-DC-SIGN interaction, interaction of *N. meningitidis* with other LPS-binding molecules seems to critically depend on inactivated bacteria since no DC association was found for live wildtype *lgtA* or *galE* mutant bacteria. These molecules remain to be identified, although likely candidates include the mannose receptor and other members of the C-type lectin family.

The T cell responses driven by DCs pulsed with the *lgtB* mutant showed a marked shift in the Th1 versus Th2-cell balance towards Th1. Recent studies on *H. pylori* demonstrated binding of DC-SIGN by bacteria expressing Le^X-LPS to reduce Th1 cell induction of naive T cells by DCs[22]. Moreover, this bias towards Th2 paralleled an increase in IL-10 production. In contrast, the Th1 response found with the *lgtB* mutant did not correlate with a skewed cytokine balance towards IL-12. However, IL-12 production is not obligatory for Th1 polarization, since IL-12 deficient mice are still capable in developing Th1 cells[23]. Thus, targeting of DC-SIGN can modulate the immune response in either direction. The mechanisms via which DC-SIGN directs T cell proliferation are currently unknown. It has been suggested that the polarization may depend on the balance between activation of CLR and TLRs[24]**.** *N. meningitidis* and *H. pylori* exhibit different activity towards TLR4[25-27] and it can be speculated that this difference explains the diverse T cell proliferation and cytokine response induced by these organisms.

A typical feature of *N. meningitidis* LPS is the phase variable expression of the terminal oligosaccharide structure. This is caused by slipped-stranded mispairing of homopolymeric G tracts within the 5'-coding sequence of the *lgt* genes encoding glycosyltransferases that sequentially and specifically add sugar residues to the growing oligosaccharide chain. The assembly of the lacto-*N*-neotetraose unit is genetically determined by the *lgtA*, *lgtB*, and *lgtE* genes, of which only *lgtA* can undergo phase variable expression leading to loss of the lacto-*N*-neotetraose unit, and a switch from immunotype L3 to L8[28]. In agreement with this hypothesis is the rare occurrence of the immunotype L6 in clinical isolates[11], which expresses the same terminal GlcNAcβ1-3Galβ1-4Glc-R outer core trisaccharide as the *lgtB* mutant of H44/76, and, as our present data indicate, has the same specificity for DC-SIGN.

Moreover, no *lgtB* negative strain was found in a collection of 37 strains belonging to the major meningococcal genetic lineages[29]. These observations demonstrate how minor variations in meningococcal LPS structure can profoundly influence the specificity and outcome of the host-pathogen interaction.
Finally, our data demonstrate that *lgtB* LPS represents the first, and possibly unique, defined molecule that upon DC-SIGN targeting forces DCs into a T helper 1 mode. This finding has important implications for future vaccine development and immunotherapy. Targeting of DC-SIGN with LPS derivatives expressing the GlcNAcβ1-3Galβ1-4Glc-R oligosaccharide core structure facilitates direct uptake of antigens by DCs, thereby conceivably generating a more effective immune response and increasing vaccine efficacy. In particular, the combination of the *lgtB* mutant oligosaccharide with the less toxic *lpxL1* penta-acylated lipid A[30] might lead to an effective adjuvant.

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CHAPTER 11

DISCUSSION

MGL: THE ODD ONE OUT?

In this thesis the characterization of the carbohydrate recognition profile and functions of the human C-type lectin MGL is described. MGL is preferentially expressed by antigen presenting cells (APCs) that possess tolerogenic properties, such as dexamethasone-cultured dendritic cells (DCs) or alternatively activated macrophages (Chapters 2 and 4)[1,2]. Furthermore, MGL is involved in several aspects of the immunobiology of APCs, including cell-cell adhesion, pattern recognition, antigen uptake and presentation, and immunomodulation of interacting effector T cells (summarized in fig. 1)[3]. When comparing our data to results reported for other C-type lectins, the question arises how functionally different or similar is MGL compared to other APC-expressed C-type lectins? It is therefore interesting to evaluate some of the functions performed by MGL in relation to other APC-expressed C-type lectins. MGL has a unique and exclusive carbohydrate specificity for terminal GalNAc structures, such as the Tn antigen or the LDN epitope (Chapter 3)[3]. Although the C-type lectin ASGP-R and the related galectins are able to recognize these glycans, they have an intrinsic specificity and preference for galactose, suggesting that these lectins bind a much broader array of ligands and possess additional functions[4,5]. In contrast to ASGP-R and the galectins, MGL is only expressed by APCs, suggesting that MGL mediates recognition of terminal GalNAc structures by these cells. APCs probably express another lectin with GalNAc specificity. The identity and full recognition profile of this lectin is currently

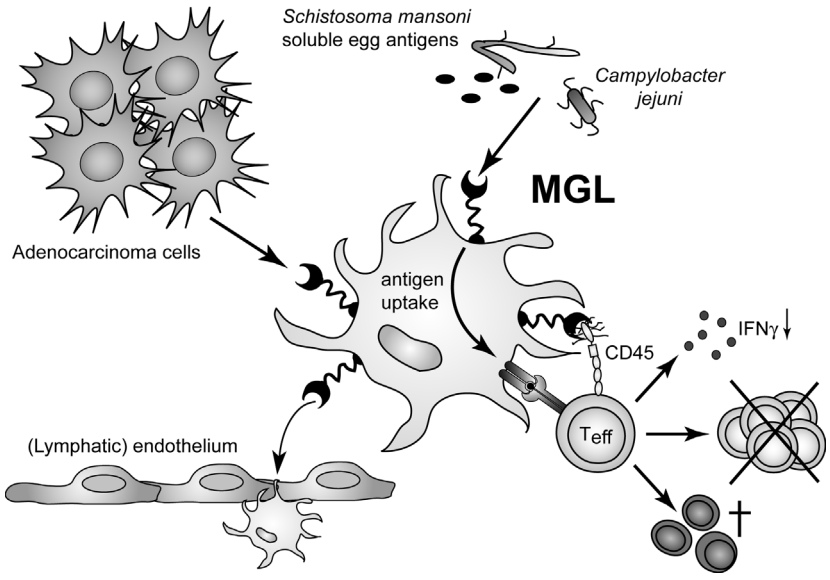

Figure 1. MGL-mediated functions. The C-type lectin MGL mediates specific recognition of tumor cells and binding and uptake of pathogens, such as *Schistosoma mansoni* and *Campylobacter jejuni*. Endocytosis via MGL can result in presentation of antigenic peptides in MHC class II. Furthermore, MGL mediates retention of immature MGL$^+$ APCs in skin, lymph node and thymus and facilitates negative regulation of effector T cell activation under steady state conditions or during chronic inflammation.

unknown and may also include galactose-type structures (Chapters 3 and 5)[3]. Of course, APCs express a wide variety of mannose-type lectins, such as DC-SIGN and mannose receptor (MR), that together cover an extensive range of high mannose and Lewis antigen-containing ligands[6,7]. Clearly, MGL shares quite some general characteristics with these APC-expressed C-type lectins. However, the restricted and distinctive specificity of MGL suggests that MGL might also confer exclusive, MGL-specific functions to APCs.

General functions of C-type lectins: cell-cell adhesion

Many C-type lectins function as adhesion or homing receptors by binding to cellular counter-receptors or extracellular matrix proteins. Several C-type lectins, including langerin, Endo180 and MR interact with collagen components, potentially enabling APCs to migrate into the tissue or to facilitate local APC retention[8-10]. Especially during trafficking of leukocytes C-type lectins appear to be involved. MR on lymphatic endothelial cells contributes to lymphocyte exit from lymph nodes by binding to L-selectin on the lymphocyte surface[11]. DC-SIGN binding to endothelial ICAM-2 mediates recruitment of DC precursors to the peripheral tissues[12]. Similarly, the C-type lectin family of selectins facilitates lymphocyte homing and entry into lymphoid tissues and sites of inflammation[13]. Human MGL specifically interacts with GalNac structures in the skin and on sinusoidal and lymphatic endothelial cells in LN and thymus. This interaction does not facilitate migration; instead MGL^+ APCs are locally retained in the tissue (Chapter 5). Since MGL expression is lost upon maturation, mature APCs will be released from GalNAc constraints and can initiate advanced migration. In contrast to the abundantly expressed sialylated Lewis antigens that bind to selectins, MGL recognizes more restricted terminal GalNAc moieties. These terminal GalNAc structures are much less common and probably these epitopes are expressed only at certain sites in the body or at certain time points during inflammation[14]. For example, the MGL-binding LacdiNAc epitope (LDN or GalNAcβ1-4GlcNAc) is specifically upregulated on endothelial cells after activation by the proinflammatory cytokine TNFα through specific upregulation of the β4GalNAc-transferase in these cells[15].

General functions of C-type lectins: pattern recognition and antigen presentation

Pattern recognition is often named as a general function of C-type lectins. According to the definition a true pattern recognition receptor alerts the innate immune system to the presence of incoming pathogens[16]. In this context, Dectin-1 fits all criteria with specific recognition of several fungal species and subsequent production of reactive oxygen species and proinflammatory cytokines[17,18]. Although C-type lectins, such as DC-SIGN and MR, interact with an extensive variety of pathogens, only three pathogens have been identified so far that specifically interact with MGL, namely the filoviruses[19], the helminth parasite *Schistosoma mansoni* (Chapters 3 and 9)[3] and the bacterium *Campylobacter jejuni* (Chapter 8). It is currently unclear whether binding to MGL, DC-SIGN or MR contributes to anti-microbial immune responses or whether

the pathogens target these lectins for their immunoregulatory properties in favor of pathogen survival (see below)[20,21].

In addition to cell-cell adhesion and pattern recognition, receptor-mediated endocytosis of (self-)antigens or pathogens for presentation in MHC class I/II or CD1 molecules appears to be a general feature of C-type lectins. So far, all C-type lectins studied seem to be capable of internalizing after ligand binding or antibody crosslinking, even though some of them lack clear consensus internalization motifs in their cytoplasmic regions[22,23]. Endocytosed structures, proteins or pathogens are subsequently guided along the endosomal/lysosomal pathway for degradation and antigen presentation. The growing list of C-type lectins and C-type lectin-like molecules suitable for efficient delivery of antigens for presentation, now includes MR, DEC-205, DC-SIGN, langerin, Dectin-1, Dectin-2 and BDCA-2[24-31]. Also MGL is capable of internalizing ligands for MHC class II presentation (Chapter 6). The YENF motif, matching the YXXØ consensus motif[32], in the MGL cytoplasmic domain is indispensable for this process. However, MHC class I and/or class II presentation is no guarantee for immune activation. Elegant experiments using DEC-205 antibodies coupled to ovalbumin (OVA) clearly demonstrated that this mechanism of antigen delivery did not result in long-term immunity, instead responder T cells are deleted from the repertoire[25]. In addition, induction of regulatory T cells was observed[33]. When the DEC-205-OVA complexes were administered together with a 'danger' signal, e.g. an agonistic anti-CD40 antibody, a full-blown effector-type $CD4^+$ and $CD8^+$ T cell response was generated[34]. Thus, the fact that many C-type lectins bind to self-glycoproteins and the absence of immune activation after C-type lectin targeting, led to the assumption that C-type lectins do not primarily function as pattern recognition receptors. Instead they internalize self-antigens for presentation in steady state, thereby actively contributing to homeostasis control[35]. This hypothesis is supported by recent findings in the MR knockout mouse. These $MR^{-/-}$ mice show no differences in immunity to pathogens such as *Candida albicans* and *Pneumocystis carinii*. They do accumulate several endogeneous glycoproteins in the serum, due to a lack in clearance of these molecules[36-38]. Further evidence comes from the evolution of the human DC-SIGN gene that has been under a strong selective constraint that prevents accumulation of any amino acid changes, signifying that DC-SIGN has a certain crucial function and thus is not subject to pathogenic pressure[39]. Although endocytosis of pathogens via C-type lectin-mediated pathways can result in degradation and presentation of specific microbial peptides or lipids[40,41], several pathogens target C-type lectins to escape immunosurveillance and to promote their own survival by reducing the powerful antigen-presenting capacities of DCs and the modulation of naive T cell priming (see below)[20,42-44]. Strikingly, some DC-SIGN polymorphisms are associated with a decreased susceptibility to tuberculosis, suggesting that during certain infections, C-type lectins do confer immune protection[45]. Further studies are required to determine whether MGL can function as a bona fide pattern recognition receptor and whether MGL polymorphisms or expression levels are correlated with disease outcome in helminth parasite or

Campylobacter jejuni infections.
In conclusion, cell-cell or cell-matrix interactions and antigen uptake for processing and presentation appear to be general functions of the C-type lectin family. But what is the distinguishing factor between the many different C-type lectins? Each lectin does possess its own individual carbohydrate recognition profile; nevertheless there is quite some redundancy in the structures that are recognized. Especially high mannose-type glycans are bound by several C-type lectins, including the MR, DC-SIGN, L-SIGN, langerin and Dectin-2. It is my opinion, that the differential signaling upon ligand binding is what differentiates C-type lectins from each other. Combined with the selective expression of the different C-type lectins in separate APC subsets (Chapter 2)[1], the differential signaling pathways provide a strong mechanism for fine-tuning immune responses.

C-type lectin-mediated immunomodulation: tyrosine-based activation or inhibitory motifs

Interactions between C-type lectins and their ligands can potentially modulate both the host cell and the ligand-expressing cell. So, specific signaling can be derived directly from the ligand or be induced by the C-type lectin itself. Strikingly, whereas MGL is involved in regulation of the target effector T cell that harbors the MGL ligand on CD45 (Chapter 4)[2], most C-type lectins actually modify the immunostimulatory capacities of the cell they are expressed in, which often is an APC (Fig. 2 represents an overview of C-type lectin-mediated immunomodulation). Combined, the differential expression of C-type lectins in various immune cells and the great variation in the length and the amino acid composition of the cytoplasmic domains create a platform for C-type lectin-specific signaling and custom-based programming of immune responses.
Some C-type lectins possess classical activation or inhibitory motifs in their cytoplasmic tails and are thus capable of direct triggering of intracellular signaling pathways. In contrast, other C-type lectins appear to be capable of modulating host cell function in the absence of any obvious consensus sequences for signaling. Classical motifs that are present in C-type lectin cytoplasmic regions include immunoreceptor tyrosine-based activation motifs (ITAM or YxxI/L$x_{(6–8)}$YxxI/L), and immunoreceptor tyrosine-based inhibitory motifs (ITIM or I/L/VxYxxL/V). The C-type lectin DCIR contains a functional ITIM within its cytoplasmic domain and is proposed to repress cellular activation of neutrophils through a mechanism involving the phosphatase SHP-2[46,47]. Upon GM-CSF stimulation of neutrophils, SHP-2 translocates from DCIR, a cellular repressor, to the GM-CSF receptor, the cellular activator of neutrophils. The functional ITIM motif in the cytoplasmic region of DCAL-2 is thought to negatively regulate monocyte and neutrophil activity[48].
Recognition of several fungal species, including *Aspergillus fumigatus*[49] and *Candida albicans*[50], by DCs is mediated by the β-glucan receptor Dectin-1. Binding of fungal β-glucans to Dectin-1 can directly or in synergy with TLR-2 trigger secretion of cytokines, such as IL-10, TNFα and IL-12[51-54]. Both pathways depend on the

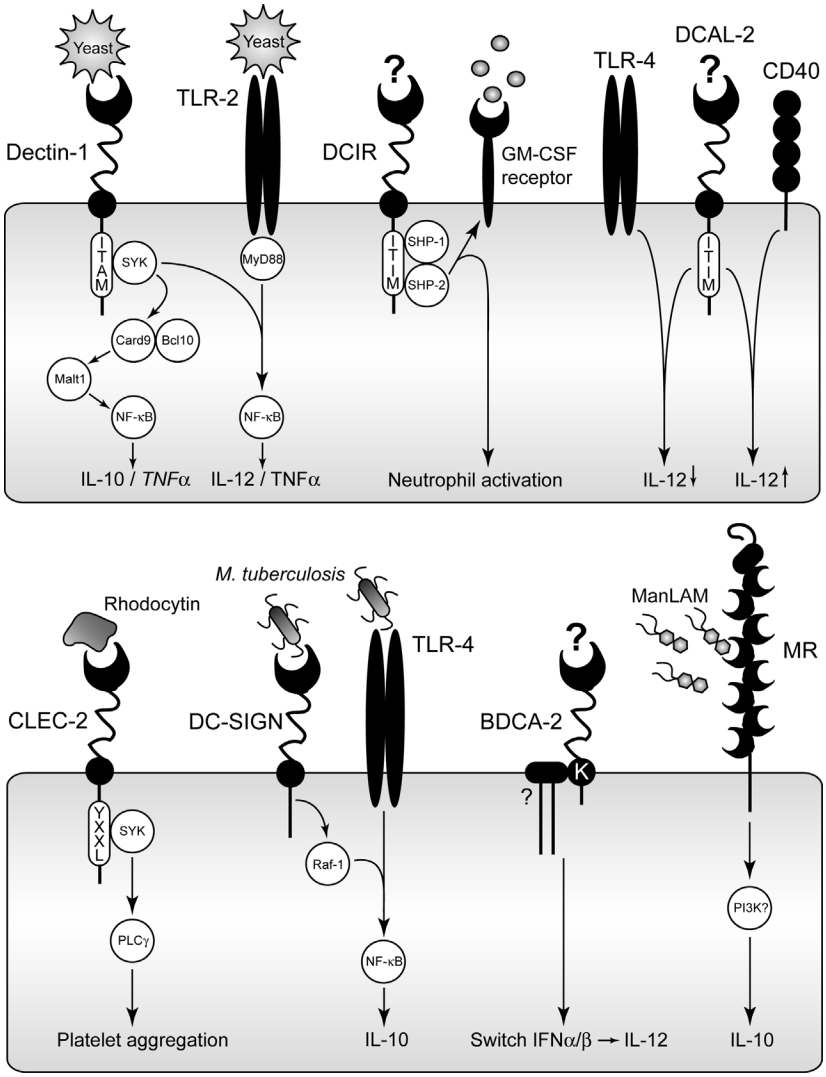

Figure 2. C-type lectin-mediated signal transduction.

functional ITAM-like motif in the Dectin-1 cytoplasmic domain and the recruitment of the tyrosine kinase Syk[53,55,56]. Recently, the direct signaling pathway of Dectin-1 was elucidated, in which the caspase recruitment domain (CARD) protein Card9 is the key signal transducer[53]. Together with Malt-1, the Card9-Bcl10 complex controls NF-κB activation and subsequent cytokine production. Strikingly, yeast phagocytosis by Dectin-1 involves Syk-independent mechanisms[57]. CLEC-2-mediated activation and aggregation of platelets by the snake venom rhodocytin is likewise dependent on Syk[58]. Upon rhodocytin binding CLEC-2 triggers tyrosine phosphorylation of the cytoplasmic YxxL motif, which facilitates recruitment of Syk and downstream activation of PLCγ2. Although DC-SIGN also harbors the YxxL motif, Syk is not involved in the DC-SIGN-induced signaling pathway (S.I. Gringhuis and J. den Dunnen, *manuscript submitted*).

C-type lectin-mediated immunomodulation in the absence of obvious signaling motifs

Also C-type lectins, which apparently lack consensus signaling motifs in their cytoplasmic regions, can regulate maturation and cytokine profiles of APCs. Binding of DC-SIGN to the pathogenic cell wall component ManLAM of *Mycobacterium tuberculosis* blocks DC maturation and causes a strong upregulation of IL-10 production[44]. In contrast to previous reports, the DC-SIGN-ManLAM interaction does not induce ERK1/2 phosphorylation[59], instead Raf-1 phosphorylation is triggered, which combined with downstream TLR signaling results in enhanced NF-κB activation and stronger IL-10 production (S.I. Gringhuis and J. den Dunnen, *manuscript submitted*). Some ligands and antibodies to the MR also stimulate IL-10 secretion[60]. Antibody crosslinking of BDCA-2 on the plasmacytoid DCs (pDCs) cell surface markedly decreases interferon (IFN)α and β secretion, thereby promoting IL-12 production by the pDCs[30,61].

C-type lectin ligand binding and modulation of cytokine production can have direct consequences for subsequent programming of $CD4^+$ T helper responses. Evidence is accumulating that certain C-type lectins can influence the Th1/Th2 balance during the polarization phase of $CD4^+$ T cells[60]. Strikingly, DC-SIGN can promote both Th1 as well as Th2-mediated responses. A Lewis Y-positive phase variant of *Helicobacter pylori* shifts responses towards a Th2-dominated phenotype through its interaction with DC-SIGN[62], whereas an *lgtB* mutant form of *Neisseria meningitidis* targets DC-SIGN to induce a Th1-mediated profile (Chapter 10)[63]. The mechanisms by which DC-SIGN modulates both Th1 and Th2 responses are currently unknown. Some factors that might influence the outcome of DC-SIGN-mediated signaling pathways include the nature of the carbohydrate ligand or the multivalency of interacting structure. On *H. pylori* LPS DC-SIGN recognizes a Lewis antigen, whereas on *N. meningitidis* a GlcNAc residue is the interacting moiety[63]. Mutagenesis studies have shown that Lewis antigens and high mannose glycans are bound by overlapping but not identical sites within the DC-SIGN carbohydrate recognition domain (CRD)[64]. Hence, the differential recognition of GlcNAc and Lewis antigens by DC-SIGN could result in disparate stimulation of intracellular pathways. Moreover, receptor crosslinking is necessary to initiate signaling, indicating that the spacing of the carbohydrate ligands and the amount of C-type lectin crosslinking could strongly influence the strength or the endurance of the signaling cascade that is induced. Finally, the outcome of an immune response is likely to be determined by the interplay of signaling pathways, as C-type lectins are usually not the only receptors to interact with a certain pathogen. Recently, Chen *et al* demonstrated such a mechanism for the C-type lectin-like molecule DCAL-2. DCAL-2 triggering by antibodies differentially programs DCs depending on the accompanying signal. Anti-DCAL-2 can suppress TLR-induced IL-12 production, whereas it augments IL-12 secretion after CD40 ligation[65].

Thus, the balance between innate activation via TLRs or CD40 ligation, and C-type lectin-mediated pathways, whether they synergize or dampen each other, determines

the strength and quality of an immune response. It will be interesting to pursue the consequences and effects of ligand or pathogen binding to MGL on APC phenotype and function and whether MGL can likewise interfere with TLR-mediated or other activation pathways of APCs.

Modulation of target cells

In contrast to the extensive knowledge we presently have on the effect of C-type lectin triggering on APC functionality, much less is known about modification of target cells that express the ligand. So far the only publications concerning modulation of target cells address the influence of C-type lectin binding on T cell functionality. Both recombinant Dectin-1 and DCAL-1 proteins can act as co-stimulatory molecules for naive T cells, however it is unknown whether on APCs these molecules actually contribute to the activation and stimulation of T cells[66,67]. Murine Dectin-2 appears to control the generation of regulatory T cells during UV radiation-induced tolerance. The exact mechanism and the Dectin-2 ligand still remain to be determined[68].

MGL directly controls the activation status of antigen-experienced effector T cells (Chapter 4)[2], a feature that has not been demonstrated for any other classical C-type lectin. MGL binding to its counter-receptor CD45 decreases the phosphatase activity of CD45, thereby negatively regulating TCR-mediated signaling and T cell-dependent cytokine responses, which in turn decrease T cell proliferation and increase T cell death. Strikingly, although thymic output is unaltered in the mMGL1 knockout mouse, these mice have a higher number of antigen-experienced memory T cells (www.functionalglycomics.org, unpublished data)[69]. These data may indicate a common mechanism of MGL-mediated control of T cell homeostasis in both mice and man. Another cellular counter-receptor of MGL, the tumor-associated mucin MUC1, also possesses several signaling properties. Signal transduction via the MUC1 cytoplasmic tail activates transcription of various proliferative genes and confers resistance to stress-induced apoptosis[70]. Future studies may address whether MGL binding to tumor-associated MUC1 on adenocarcinoma cells can induce MUC1 signal transduction and alter tumor progression.

In conclusion, MGL shares several common denominators with other classical C-type lectins, such as its capability to target antigens for processing and presentation and the binding to self-glycoproteins, facilitating the interaction between the APC and the lymphatic endothelium. The very restricted specificity for terminal GalNAc-structures and the negative regulatory function of effector T cells have so far not been demonstrated for any other C-type lectin. In conclusion, the expression on tolerogenic APCs and the downregulation of effector T cell activity suggest a specialized role for MGL in the homeostatic control of adaptive immunity.

TERMINAL GALNAC: A SIGNAL FOR IMMUNE CONTROL?

The interactions between C-type lectins and their counter-receptors depend not only on the expression and activity of the C-type lectin receptor on the APC, but also on the accessibility of the carbohydrate ligand. Interacting cells or pathogens can actively regulate lectin binding by changing the composition of their glycosylation machinery. It is therefore interesting to have a closer look at two of the major MGL ligands, the Tn antigen and the LDN epitope.

Expression of Tn and LDN epitopes

O-glycan mucin-type linkages are initiated by the transfer of an α-GalNAc sugar by an UDP-N-acetylgalactosamine:poly-peptide N-acetylgalactoaminyl-transferase (ppGaNTase) to a serine or threonine residue, forming the so called Tn antigen or α-GalNAc-Ser/Thr (see also the appendix on glycosylation)[71]. Normally, Tn antigens are only expressed at high levels in the developing brain during embryogenesis[72]. Although the Tn epitope is unveiled in approximately 90% of all carcinomas and in certain diseases, such as Tn syndrome (see below), it is shielded in healthy and benign tumors. During O-glycan synthesis, Tn antigens are further elongated, starting with the addition of a galactose in a β1-3 linkage by the Core 1 β3-galactosyltransferase (Core 1 β3GalT). For proper function Core 1 β3GalT requires the assistance of the molecular chaperone Cosmc[73]. Despite the simple catalytic function of ppGaNTases, it is estimated that the human genome harbors 24 different ppGaNTase genes with unique tissue and spatial expression patterns[74].

Compared to the Tn antigen, the LacdiNAc (LDN) epitope or GalNAcβ1-4GlcNAc is less rare in the healthy human body. Nevertheless, it is not expressed at very high frequencies. Terminal LDN structures have been detected on the human pituitary hormones lutropin and thyrotropin[75], urinary kallidinogenase[76] and on glycodelin-A, a major immunosuppressive glycoprotein in amniotic fluid[77]. Furthermore, LDN expression can be detected on human umbilical vein endothelial cells[15]. Two human glycosyltransferases, β4GalNAc-T3 and β4GalNAc-T4, facilitate the addition of a GalNAc residue in a β1-4 linkage to the GlcNAc substrate in complex oligosaccharide chains[78,79]. Helminth parasites, such as *Schistosoma mansoni*, *Haemomonchus contortus*, *Fascida hepatica* and *Dirofilaria immitis*, carry abundant LDN determinants on their glycoproteins and glycolipids[80].

Immunogenicity of GalNAc-containing epitopes

Oligosaccharides have long been described as T cell-independent antigens, generating only a characteristic primary humoral IgM repsonse, rare IgG class switching and no immunological memory. Vaccination studies have demonstrated that when poorly immunogenic polysaccharides are conjugated to highly immunogenic carrier proteins, like diphtheria toxin, strong protective humoral responses can be generated that protect against infection with pathogens that harbor those polysaccharide antigens[81]. Zwitterionic polysaccharides from the capsules of

certain bacteria, such as type 1 *Streptococcus pneumoniae* and *Bacteroides fragilis*, prove an exception to this rule. These bacterial polysaccharides are taken up and processed by the APC and directly presented in MHC class II, thereby leading to the activation of $CD4^+$ helper T cells in a glycan-dependent manner[82].

Glycosylation is a common posttranslational modification, however evidence for processing and presentation of glycosylated peptides to T cells is limited[83]. Some naturally processed glycopeptides have been eluted and sequenced from both MHC class I and MHC class II molecules[84,85]. Most of these glycopeptides contain the O-β-GlcNAc substitution, a modification often found in cytosolic proteins. Because of steric hindrance only small glycans, either mono- or disaccharides, can be accommodated within the MHC class I groove or T cell receptor and thus contribute to the specificity to the $CD8^+$ T cell response[86]. In contrast, the MHC class II groove is open at both ends and can hold a much broader array of peptide lengths and larger glycan modifications[87]. In addition to naturally processed peptides, active immunization with synthetic glycopeptides elicits protective cellular responses specific for the glycan or the whole glycopeptide. Such studies demonstrate that glycopeptide-specific T cell receptors exist within the T cell repertoire and that the carbohydrate moiety forms an intrinsic part of the antigenic determinant[88].

Thus, cellular immunity exists or can be induced towards glycopeptides, but are Tn and LDN epitopes likewise recognized as foreign structures and do they elicit humoral or cellular immune responses? All humans possess pre-existing antibodies, predominantly of the IgM subclass, to Tn antigens. The origin of these antibodies has been linked to the presence of the *Escherichia coli* O86 in the gastrointestinal tract[89]. These Tn antibodies may aid in the immune response to Tn positive carcinomas, however they are insufficient to provide full tumor protection. Although there is no pre-existing cellular immunity to Tn antigen[90], vaccines composed of designer glycopeptides with Tn antigen substitutions are able to prime Tn-specific cytotoxic T cells (CTLs), which recognize a variety of tumor cells expressing Tn antigen in the context of endogeneous glycoproteins (see also the section on Tn antigen and cancer)[91].

The LDN epitope is a self-carbohydrate, yet during helminth infections high levels of protective anti-glycan antibodies are generated, suggesting that tolerance to LDN is not absolute[80]. Clearly, the highly immunogenic nature of the parasitic glycoproteins is enough to induce a humoral response. Probably, the amount and spacing of LDN structures on the parasitic glycoproteins is very different compared to host glycoproteins, thereby creating completely novel glycan epitopes. In chapters 3 and 9 we have demonstrated that immature DCs can recognize *Schistosoma mansoni* soluble egg antigens (SEA) through the interaction with MGL, MR and DC-SIGN[3]. These three lectins cooperate in the endocytosis of the SEA, potentially leading to MHC class II presentation of parasitic (glyco)peptides to the immune system and the generation of humoral immunity.

During the reproductive cycle of the *S. mansoni*, worms mate and produce eggs, which become lodged in host tissues, such as the liver, where they cause

granulomatous inflammation and organ damage. Strikingly, hepatic implantation of LDN-coated beads trigger granuloma formation similar to the lesions found in natural schistosome infection[92]. Galactin-3 has been implicated in granuloma induction[93], still MGL might equally participate in this process. In mice, alternatively activated macrophages initiate granuloma formation[94], a cell type that expresses elevated levels of mMGL[95]. In addition, mMGL1 is involved in other antigen-dependent mouse models of granuloma formation. In an air pouch model mMGL1$^+$ macrophages secrete the proinflammatory cytokine IL-1α and thereby regulate the cellular influx and fibroblast activation necessary for tissue remodeling[96,97]. Moreover, the induction of such granulomatous inflammation was not observed in mice which lack mMGL1 expression[96]. The formation of egg granulomas in *S. mansoni* infection is a Th2-mediated process[98]. Strikingly, IL-1α is essential for the protective Th2 switch during parasite infection[99]. Therefore, mMGL$^+$ macrophages may actively contribute to egg-induced tissue remodeling.
In conclusion, both the Tn antigen and the LDN epitope can elicite both humoral and cellular responses, provided they are coupled to immunogenic or foreign proteins. Antibodies and CTLs to Tn antigens may contribute to anti-tumor immunity, whereas LDN glycans can trigger granuloma formation, a host defense mechanism against persistent antigens or infection.

Glycosylation and immune disease
In addition to a broad range of carcinomas, Tn expression has been reported in a variety of rare human diseases such as Schindler-Kanzaki disease, a lysosomal storage disease[100], IgA Nephropathy (IgAN)[101], Henoch-Schonlein purpura[102] and Tn syndrome[103]. So far, no diseases have been described in which expression of the LDN epitope is elevated or altered. Because of the increased exposure Tn epitopes, MGL may be involved in the pathogenesis of both IgAN and Tn syndrome.
In Henoch-Schonlein purpura and IgA Nephropathy (IgAN) the hinge region of serum IgA is aberrantly O-glycosylated, displaying a reduced level of galactosylation and thus an increased level of Tn antigen. N-glycosylation appears normal[102,104]. The changes in O-glycosylation have been linked to a decreased expression of Cosmc in B cells[105]. IgAN mainly affects the kidneys where a strong mesengial deposition of IgA immune complexes is observed, which leads to inflammatory glomerular damage and possibly renal failure[106]. Normally, IgA is cleared in the liver by the hepatic C-type lectin asiologlycoprotein-receptor (ASGP-R), which recognizes the terminal galactose residues in the O-glycans located in the hinge region[107]. The reduced galactosylation could impair the binding and uptake of IgA by ASGP-R, facilitating the persistence of aberrant IgA in serum. Furthermore, the increased exposure of Tn antigens on IgA might trigger enhanced recognition by MGL on APCs. Through MGL-mediated endocytosis the deviant IgA could be processed and presented to the immune system (Chapter 6), providing a mechanism for the existence of IgG auto-antibodies to the GalNAc glycans and hinge region of IgA in IgAN[108]. Thus, MGL might contribute to disease progression in IgAN.

Tn syndrome is a rare hematological disorder characterized by the expression of the Tn antigen on all leukocyte lineages[103]. The disease is caused by a mutation in the Core 1 β3GalT chaperone Cosmc in pluripotent stem cells, resulting in increased exposure of Tn antigen on subpopulations of blood cells[109,110]. Thus, in Tn syndrome normal cells co-exist with affected cells. Strikingly, only around 1-2% of T cells are affected, whereas approximately 50% of the B cells are transformed. It is tempting to speculate that affected T cells are recognized and selectively killed off through an MGL-CD45-dependent mechanism. Likely, the transformed T cells closely resemble Jurkat T cells, which share the Cosmc gene mutation[73]. This mutation will lead to increased exposure of Tn antigens on glycoproteins, such as CD43 and CD45. High affinity binding of MGL to CD45 on the affected T cells may thus initiate a similar apoptotic pathway as observed in Jurkat cells, resulting in enhanced death of the mutated T cells (Chapter 4)[2].
In general, host glycosylation patterns are frequently altered in immune diseases and aberrant glycosylation has been linked to autoimmunity. Knockout mice for the glycosidase α-mannosidase II develop kidney inflammation resembling the lupus nephritis observed in human systemic lupus erythematosus[111]. Similarly, β–1,6-N-acetylglucosaminyl-transferase V (GlcNAcT-V) knockout mice also acquire an autoimmune kidney disease and have an increased susceptibility to experimental autoimmune encephalomyelitis, due to an increased T cell receptor clustering that lowers the threshold for activation[112,113]. Furthermore, the activity of GlcNAcT-V is decreased by 25–30% in PBMCs of Multiple Sclerosis (MS) patients when compared to healthy controls, suggesting that also in human MS glycosylation is dysregulated[115]. Lastly, the predominant T cell epitope from collagen type II is posttranslationally modified under normal conditions and a lack of epitope glycosylation is related to rheumatoid arthritis progression[114]. In their review 't Hart *et al* propose a mechanism in which immunological tolerance to self-antigens is maintained through the balance between TLR and C-type lectin-mediated signaling in DCs[115]. A disruption in glycosylation and thus C-type lectin recognition can disrupt this delicate balance and result in autoimmune disease.

Tn antigen and cancer
Many tumor cells have altered biosynthetic pathways of mucin-type O-glycans due to aberrant expression levels and activities of glycosyltransferases, resulting in the over- or underexpression of naturally occurring glycans or re-expression of embryonic carbohydrate structures[116,117]. Several carbohydrate structures, such as the Tn antigen, sialyl-Tn (Sialylα2-6GalNAcα-Ser/Thr), Thomsen-Friedenreich antigen (TF antigen or Galβ1-3GalNAcα-Ser/Thr) and (sialylated) Lewis antigens, are elevated in malignant cells[118]. These tumor-related glycan antigens are potential ligands for C-type lectins on APCs[119], whereby only the Tn antigen binds with high affinity to human MGL (Chapter 3)[3]. Unlike the tissue-specific tumor-associated protein antigens, Tn antigen is broadly expressed in over 90% of all carcinomas, including breast, ovary, colorectal, gastrointestinal, bladder, larynx and lung

carcinomas[116]. Moreover, Tn antigen is already detectable at early stages of tumor development. The occurrence of Tn antigen is associated with invasive and highly proliferative tumors, metastasis and poor clinical outcome[118].

One of the best-studied O-glycosylated proteins in tumor cells that displays the Tn antigen, is the epithelial mucin MUC1. During malignant transformation MUC1 levels are increased and its polarized apical expression is lost. Furthermore, in adenocarcinoma MUC1 is aberrantly glycosylated, resulting in increased exposure of shorter tumor-associated glycans, such as Tn and TF antigens[120]. In MUC1-deficient mice mammary tumors display a delayed growth, demonstrating that the aberrant MUC1 is important for either tumor progression or tumor metastasis[121]. The transformed expression, combined with a low immunicity of MUC1 in adenocarcinoma patients, indicates that MUC1 might be a promising target for immunotherapy of cancer patients. In this thesis we have demonstrated that MGL can specifically recognize the tumor-associated form of MUC1, distinguishing it from its normal counterpart (Chapter 7). During processing of MUC1 glycoprotein, carbohydrates are not efficiently removed and glycopeptides are loaded onto MHC molecules[122,123]. Therefore, MGL targeting and endocytosis of tumor-derived Tn positive MUC1 might result in processing and presentation of MUC1 glycopeptides to the immune system.

Theoretically, an ideal tumor vaccine should provide both cytotoxic $CD8^+$ T cell immunity, as well as therapeutic antibodies, which fixate complement or facilitate antibody-dependent cellular cytotoxicity (ADCC). Already in 1991, Singhal *et al* demonstrated that immunization of mice with desialylated bovine submaxillary mucin, which contains a high amount of Tn antigen, could protect against a subsequent challenge with a highly invasive syngeneic tumor[124]. The observed immunity was glycosylation-dependent as immunization with the deglycosylated mucin could not provide protection. Furthermore, in non-human primates, a newly developed synthetic dendrimeric Tn antigen vaccine gives rise to tumor-specific antibodies that mediate ADCC against human tumors[125].

Several studies in mouse models of adenocarcinoma have evaluated the potential of MUC1 as a tumor vaccine, especially in mice transgenic for the human MUC1 gene. These mice are functionally tolerant to the MUC1 protein and develop more progressively growing MUC1 positive tumors compared to wildtype mice[126]. An immunization strategy with DC-tumor cell fusions efficiently primed both $CD4^+$ and $CD8^+$ T cell responses and resulted in rejection of established metastasis in transgenic mice, without any apparent autoimmunity to non-transformed normal epithelia[127,128], showing the potential feasibility of targeting MUC1 for active immunotherapy.

Also in human phase I or II clinical trials the potential of MUC1 as a therapeutic agent has been assessed, in which different strategies, including DNA vaccination, mannan-conjugated MUC1, peptide pulsed DCs or MUC1-transfected/transduced DCs, have been employed[129]. Vaccines based on *ex vivo* peptide loading of DC show enhanced delayed-type hypersensitivity (DTH) responses, some induction of CTL responses and at best partial remissions in a subgroup of patients with advanced

staged cancer[130-133]. However, in all these studies naked peptides were used, capable of generating only peptide-specific responses without additional carbohydrate-specific responses that might be elicited, if glycopeptides were included in the vaccine. Therefore, the use of MUC1-transfected DCs might be a better and more efficient option. Although mature DCs express the mucin, MUC1 overexpression results in aberrantly glycosylated MUC1 at the cell surface, displaying Tn and TF antigens, similar to tumor-derived MUC1[134]. A fraction of these partially glycosylated MUC1 proteins could be degraded and presented as glycopeptides to $CD8^+$ T cells. Unfortunately, so far results with RNA or DNA transfected DCs were similar to those obtained with peptide-pulsed DCs, giving rise to enhanced DTH and CTL responses in a subgroup of patients with advanced cancer[135,136]. In conclusion, Tn positive tumor antigens, such as MUC1, are good candidates for inclusion in a therapeutic tumor vaccine. Preferably, such a vaccine should combine both protein and carbohydrate antigens to ensure the generation of full anti-tumor immunity and contain a maturational stimulus or adjuvant to overcome any tolerizing signals that might originate from the C-type lectins that interact with the glycosylated tumor antigens[119].

Terminal GalNAc: a signal for immune control?

One question that still remains is whether rare terminal GalNAc epitopes deliver a signal to the innate or adaptive immune system for danger or immune control. To try and answer that question, the current knowledge on MGL function and the expression of terminal GalNAc structures are combined in the setting of a MUC1-Tn antigen positive tumor and an MGL-expressing APC (Fig. 3).

In mice, mMGL-transfectants specifically home to metastatic tumor sites through the recognition of tumor-associated glycans[137]. A similar recruitment might occur in human Tn^+ tumors, as we observed clear infiltration of MGL^+ APCs in human colon cancer tissue as well (Chapter 7, Fig. 3.1). Under the influence of the tumor microenvironment local macrophages can develop into polarized M2 or alternatively activated macrophages that secrete high amounts of IL-10 and promote tumor growth[138]. Also DCs that differentiate inside a $MUC1^+$ tumor acquire a tolerogenic phenotype, characterized by high IL-10 production and low expression of costimulatory molecules[139,140]. Strikingly, tolerogenic APCs express increased levels of MGL on their cell surface (Chapter 2)[1]. Tumor loads significantly increased when mice were injected with anti-mMGL antibodies, indicating that mouse $mMGL^+$ APCs indeed contribute to anti-tumor immunity (Fig. 3.3)[141]. The mechanism for this observed anti-tumor effect is unknown and could involve both uptake and presentation of tumor-associated antigens to T cells as well as a direct inhibitory effect on the tumor itself, as a functional consequence of the mMGL-tumor interaction. A positive contribution of human MGL to anti-tumor immunity does not seem very plausible, since MGL is mainly expressed by more tolerogenic-type APCs that express low levels of costimulatory molecules. Inside the tumor, MGL^+ APCs can take up tumor material for processing and presentation to T cells (Chapter 6).

However, without an accompanying maturational stimulus, antigen presentation via C-type lectins does not result in immune activation, instead it leads to the deletion of responder T cells or the induction of regulatory T cells[25,35,139], which will suppress any further anti-tumor responses (Fig. 3.2). Furthermore, the tumor-microenvironment can actively suppress the immune stimulatory capacities of DCs. Tumor-derived

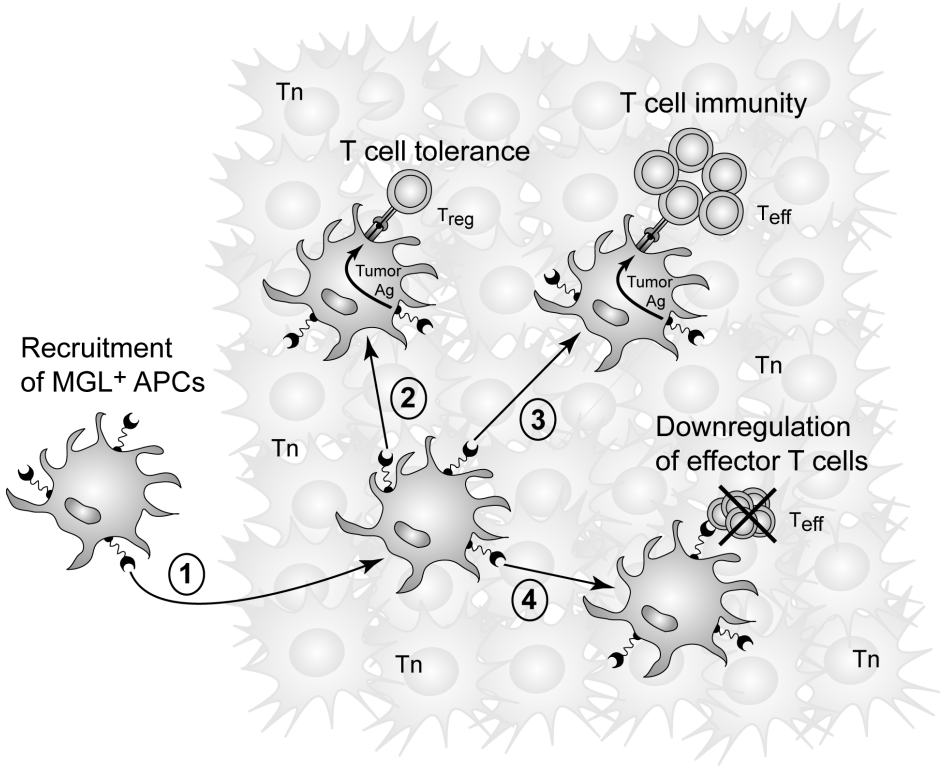

Figure 3. Model of the interaction of MGL expressing APCs with Tn positive tumors. (1) MGL$^+$ APC will be recruited to the tumor sites expressing Tn antigen. Possible consequences of this recruitment include: (2) MGL-mediated uptake of tumor glycoproteins in the steady state leading to presentation of tumor antigens and induction of T cell tolerance, (3) MGL-mediated uptake of tumor glycoproteins in combination with proinflammatory stimuli, such as necrotic tumor tissue, resulting in the induction of effector T cell immunity, and (4) MGL-mediated downregulation of tumor-specific CTLs.

MUC1 instructs DCs to drive Th2–mediated responses in contrast to Th1 immunity important for tumor eradication[142]. Even though MGL$^+$ APCs could endocytose tumor fragments, they might be unable to exit the tissue to migrate to the lymph node. Due to the high affinity MGL-Tn antigen interaction MGL$^+$ APCs will probably be retained inside the tumor (Chapter 5), where they can subsequently downregulate incoming tumor-specific cytotoxic T cells in an antigen-specific manner. The interaction of MGL with CD45 on effector T cells negatively regulates T cell receptor signaling and T cell activation (Chapter 4)[2]. In a mouse model of pancreatic cancer, activated MUC1-specific CTLs infiltrate the tumor, where they become anergic and tolerant to their antigen[143]. Although this immunosupression was partially attributed to IL-10 secretion by the tumor, other mechanisms, such as MGL-mediated downmodulation of effector T cell activation may contribute to the effect.

In conclusion, the current knowledge on MGL function and the fact that Tn positive tumors have a poor prognosis, establish that Tn antigens do not function as classical danger signals capable of alarming the immune system. In my opinion, terminal GalNAc structures might provide a signal for immune control. Several lines of evidence support this hypothesis. The relatively rare expression of these sugars may be indicative of highly specialized functions; an example is the expression of the LDN epitope on glycodelin-A, which may be related to its immunosuppressive function on T cells[77]. Strikingly, the presence of LDN glycans on beads is enough to induce granuloma formation, a host defense response to contain and control persistent infection[92]. Effector T cells are indispensable for cellular immune responses, but their activation status needs to be actively restrained to avoid excessive inflammation, tissue damage or even autoimmunity. The specific display of terminal GalNAc structures on CD45 allows tight regulation of effector T cells by the C-type lectin receptor MGL on tolerogenic APCs (Chapter 4)[2]. Cancer cells probably exploit the expression of tumor-associated glycans as an immune escape mechanism. Through the expression of Tn antigen, MGL^+ tolerogenic APCs will be recruited, which do not induce immunity, instead peripheral T cell tolerance to the cancer antigens is maintained[144]. Local MGL^+ alternatively activated macrophages can play a central role in tumor progression, through their high tissue remodeling and angiogenic capacities[96,138]. Furthermore, MGL^+ APCs can dampen any cytotoxic responses directed at the tumor[2]. In conclusion, terminal GalNAc moieties do not signal danger but rather convey a message for immune control.

CONCLUDING REMARKS

This thesis describes the characterization of the C-type lectin MGL, which is preferentially expressed by tolerogenic APC subsets, and has a unique restricted specificity for terminal GalNAc structures. Furthermore, we explored the functional consequences of C-type lectin-pathogen interactions with regard to DC maturation and its capability to drive naive T cell differentiation. Our work provides important knowledge and insights on DC and macrophage immunobiology and may eventually lead to new therapeutical strategies in microbial infections, vaccination protocols, tumor eradication and immune intervention in autoimmune diseases or chronic inflammatory conditions.

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APPENDIX

MAMMALIAN GLYCOSYLATION

Glycosylation is the cellular process in which carbohydrates or sugar chains are attached to biological compounds such as proteins and lipids[1,2]. Glycans are assembled from building blocks of monosaccharides. The most common mammalian monosaccharides include mannose, fucose, galactose, GalNAc, glucose, GlcNAc and sialic acid. Specialized enzymes catalyze either the addition of monosaccharides, the glycosyltransferases, or the removal of sugars from the carbohydrate structure, the glycosidases. It is estimated that about 1-3 % of the total human genome is dedicated to the glycosylation machinery, defined as the set of enzymes, chaperons, regulatory molecules, co-factors, sugar-donors, and other molecules involved in glycan biosynthesis.

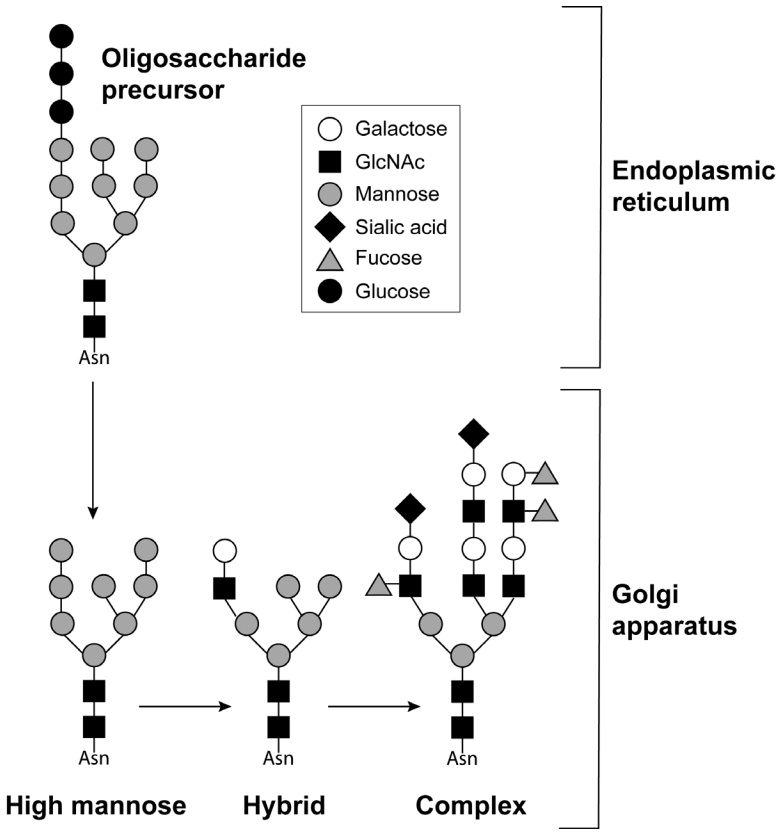

Figure 1. Schematic representation of N-linked glycosylation.

Mammalian protein glycans can be linked to asparagine (N-glycans) or to serine or threonine residues (O-glycans). N-glycosylation is initiated in the endoplasmic reticulum (ER) with the attachment of the preformed dolichol-linked precursor oligosaccharide to the asparagine in the consensus sequence, Asn-X-Ser/Thr. This precursor oligosaccharide is necessary for protein quality control and once proteins are correctly folded, they are transported to the Golgi, where glycans are further trimmed and modified. N-linked glycans can be divided into three main types,

namely high mannose, hybrid and complex-type glycans. A schematic representation of N-glycosylation is given in figure 1.
O-glycosylation is initiated in both the ER and the Golgi. The most common type of O-glycan starts with the attachment of a single α-GalNAc residue to serine or threonine. In contrast to N-glycosylation, no clear consensus sequence for O-glycosylation has been established. The single α-GalNAc residue can be further extended to form different O-glycan core structures (summarized in figure 2). Several O-glycan core structures can be elongated to generate more complex-type glycosylation. The core 1 and 2 are most abundant on leukocytes, whereas core 3 and 4 are predominantly found in the gastrointestinal tract epithelium. Mucin-type glycosylation comprises of multiple repetitive sites occupied with O-glycans. The single α-GalNAc and the core 1 structure are elevated in certain types of cancer, in which they are designated as Tn antigen and Thomsen-Friedenreich antigen (TF-antigen, Fig. 2).

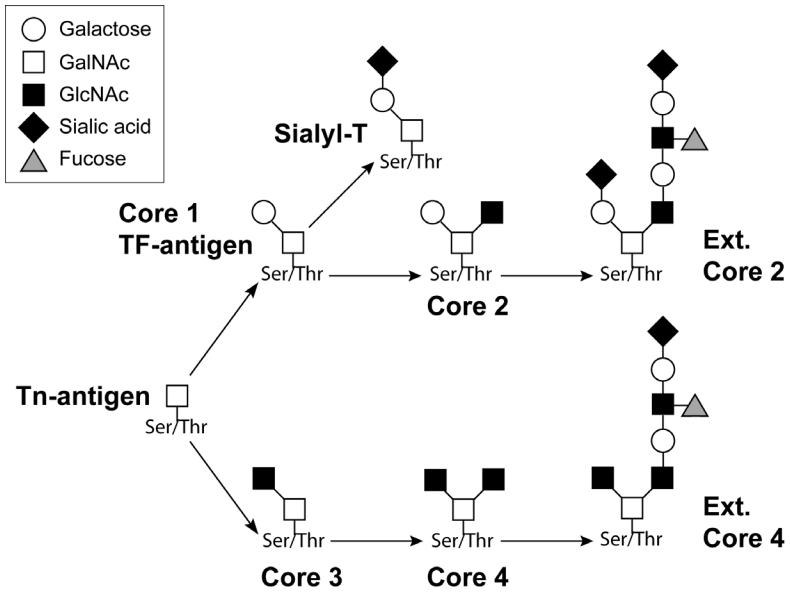

Figure 2. O-glycan core structures

Glycolipids or glycosphingolipids are abundant components of the mammalian plasma membrane that are characterized by a membrane-embedded ceramide moiety to which an extracellular carbohydrate structure is attached. Both neutral and sialylated glycolipids, termed gangliosides, exist in mammals. Glycolipids are assembled in the Golgi by the subsequent action of glycosyltransferases on the glucosylceramide core structure. Some ganglioside structures are shown in figure 3.
Glycosylation is a tightly regulated process, varying with cellular development, differentiation and activation. The importance of glycosylation is emphasized in several syndromes in which genetic mutations occur in several glycosyltransferases, glycosidases or sugar transporters involved in the glycosylation pathway. These congenital disorders of glycosylation can lead to malformations, mental retardation and even embryonic death[3]. Similarly, in chronic inflammatory conditions or cancer

glycosylation is altered[4], significantly contributing to disease progression and pathology. In conclusion, glycosylation comprises an important modification of proteins and lipids, relevant in several biological processes in health and disease.

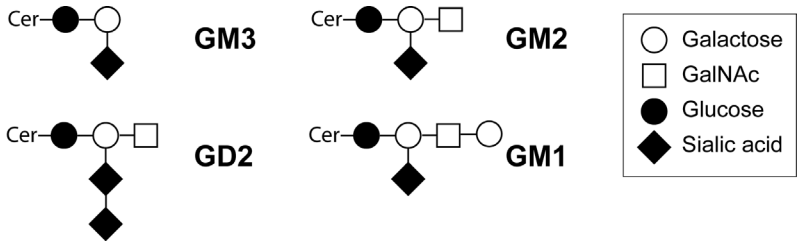

Figure 3. Examples of mammalian ganglioside structures.

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NEDERLANDSE SAMENVATTING VOOR NIET-INGEWIJDEN

Ons afweersysteem staat dagelijks bloot aan allerlei ziekteverwekkers of cellen die mogelijk in de toekomst tot tumorcellen kunnen uitgroeien. Eén van de verschillen tussen deze ziekteverwekkers of potentiële tumorcellen en onze eigen 'normale' cellen zijn de suikers die zich op het oppervlak te bevinden. De schildwachten van ons afweersysteem, de dendritische cellen, hebben speciale eiwitten ontwikkeld die deze veranderingen in de gaten kunnen houden en indien nodig een afweerreactie kunnen opstarten.

Het glycosyleringsproces

Decennia lang werden ze afgescheept als pure decoratie, de suikerketens die zich op vrijwel alle eiwitten op ons celoppervlak bevinden. Sinds een paar jaar weten we dat suikers juist hele specifieke functies vervullen binnen ons afweersysteem. Suikers zijn essentieel voor de communicatie tussen verschillende witte bloedcellen, ze regelen het verkeer van deze bloedcellen in de lymfeklieren en de milt en bovendien helpen ze ons afweersysteem verschil te maken tussen wat lichaamseigen en wat lichaamsvreemd is.

Hoe worden nu deze suikerstructuren gemaakt? In bepaalde organellen in iedere cel, zoals het endoplasmatisch reticulum (ER) en het Golgi apparaat, worden nieuw aangemaakte eiwitten 'behangen' met suikers, een proces dat glycosylering wordt genoemd. Wetenschappers hebben bepaald dat 1-3% van onze genen codeert voor enzymen, die betrokken zijn bij dit proces. Er bestaan grofweg twee vormen van eiwitglycosylering, namelijk N-gebonden en O-gebonden glycosylering. N-gebonden glycosylering wordt gestart door een voorgevormde suikerketen, die bestaat uit 9 suikers, te bevestigen aan het aminozuur asparagine. De O-gebonden glycosylering start met de aanhechting van een enkel N-acetylgalactosamine (GalNAc) suiker aan het aminozuur serine of threonine. In het Golgi apparaat worden alle N- en O-gebonden suikerstructuren verder bewerkt door een hele batterij enzymen die allemaal hun eigen stapje in de opbouw (glycosyltransferases) of afbraak (glycosidases) van deze ketens katalyseren.

Een enorme diversiteit kan worden gecreëerd doordat de 10 suikerbouwstenen in ons lichaam op verschillende punten aan elkaar bevestigd kunnen worden, waarbij niet alleen lineaire, maar ook vertakte suikerpolymeren worden gevormd. Bovendien kan de koppeling naar boven of juist naar beneden gericht zijn, wat een enorm scala aan mogelijkheden oplevert. Ter vergelijking: met de 4 basen uit het 'DNA-alfabet' zijn 256 verschillende manieren te bedenken om de 4 basen aan elkaar te koppelen. Met 20 aminozuren levert dat ongeveer 160.000 eiwit-mogelijkheden op. Maar zelfs met de simpele suikers uit ons lichaam zijn meer dan 15 miljoen combinaties mogelijk.

Elke cel, in elk weefsel en orgaan, bezit zijn eigen glycosyleringspatroon, wat kan

variëren onder invloed van de activiteit en de status van de cel. Veranderingen ontstaan door veroudering, binnendringen van ziekteverwekkers en allerlei variaties in het milieu waarin de cel zich bevindt (bijvoorbeeld hormonale veranderingen). Een tumorcel zal dus hele andere suikers op zijn oppervlak hebben dan de normale cellen waaruit de tumor ontstaan is. Ook bij zeer diverse ziektes zoals reuma en spierziektes, worden er foute suikerstructuren gevormd die bijdragen tot het ziektebeeld. Het is dus van essentieel belang om dit glycosyleringsproces in goede banen te leiden en te houden. Een belangrijke rol in de controle van al die suikerstructuren is weggelegd voor ons afweersysteem.

Het afweersysteem

Het afweersysteem kunnen we voorstellen als een leger dat een land (ons lichaam) verdedigt tegen de vijand (ziekteverwekkers en tumoren). Twee belangrijke celtypes van het afweersysteem zijn de dendritische cellen en de T-cellen, beide behorend tot de witte bloedcellen. De dendritische cellen, zogenoemd vanwege hun vele vertakkingen van het cellichaam (*dendron* is Grieks voor boom), zijn de schildwachten. Ze bevinden zich in alle weefsels, maar vooral in de gebieden die in contact staan met de buitenwereld, zoals de huid en de slijmvliezen. Ons lichaam wordt continu belaagd door indringers, zoals virussen en bacteriën, maar ook door cellen die mogelijk tot tumorcellen kunnen uitgroeien. Als dendritische cellen een potentiële vijand ontdekt hebben, slaan ze alarm en geven ze deze informatie door aan de soldaten, de T-cellen. Het doorgeven van informatie van de dendritische cellen aan de T-cellen vindt plaats in de lymfeklieren. Deze geactiveerde T-cellen beginnen vervolgens de tegenaanval om de vijand onschadelijk te maken.

C-type lectines

Dendritische cellen hebben vele herkenningsmoleculen (of receptoren) op hun oppervlak die zorgen voor de interactie met suikers. Deze specifieke suiker-herkennende eiwitten worden lectines genoemd. De grote familie van lectines bestaat uit een aantal eiwitfamilies die qua eiwitstructuur en wijze van herkenning onderling erg verschillen. De C-type lectines hebben een calcium-ion nodig om aan suikers te kunnen binden (vandaar de C). C-type lectines zijn transmembraan eiwitten die bestaan uit een korte intracellulaire (of cytoplasmatische) staart, een transmembraan domein en een rigide steel met daarop het suikerherkenningsdomein dat verantwoordelijk is voor de interactie tussen het lectine en de suiker. Lectines kunnen op allerlei cellen voorkomen, maar juist dendritische cellen hebben een aanzienlijk aantal van deze C-type lectines op hun oppervlak. In analogie met de suikers vervullen ook de C-type lectines diverse functies, bijvoorbeeld de communicatie tussen cellen maar ook de verplaatsing van witte bloedcellen door ons lichaam en de herkenning van binnengedrongen ziekteverwekkers.

Dit proefschrift

In dit proefschrift, getiteld “Nieuwe inzichten in MGL-suiker interacties in het

immuunsysteem" worden de resultaten beschreven van een promotieonderzoek naar twee C-type lectines, die veelvuldig op dendritische cellen voorkomen, namelijk het MGL en het DC-SIGN eiwit. Het suikeronderzoek in relatie tot ons afweersysteem stond bij aanvang van dit project wereldwijd nog in de kinderschoenen. Zo was er weinig bekend van het C-type lectine MGL, zelfs niet welke suikerstructuren er specifiek door deze receptor herkend werden. Om de functie van MGL goed te kunnen bestuderen, werden er monoklonale antistoffen tegen het MGL molecuul gegenereerd, zodat wij in staat waren het MGL eiwit aan te tonen op zowel geïsoleerde cellen als in humane weefsels. Zo ontdekten wij dat het MGL lectine in hoge mate aanwezig is op een aparte dendritische cel fractie, die niet zorgt voor het opwekken van een immuunreactie, maar die juist immuunreacties onderdrukt, de zogenaamde tolerogene dendritische cellen (Hoofdstuk 2). Tevens hebben wij de suikerspecificiteit van MGL geanalyseerd met behulp van een suikerchip. Dit is een klein glaasje of plaatje, waarop honderden verschillende suikerpolymeren in kleine hoeveelheden aangebracht zijn. Het MGL lectine werd door ons aan de chip toegevoegd en er werd bekeken aan welke suikers het MGL molecuul specifiek kon hechten. Op deze manier konden wij aantonen dat MGL alleen suikerstructuren herkent die een eindstandige GalNAc suiker bevatten. Deze structuren komen sporadisch voor in het menselijk lichaam, maar zijn erg talrijk op tumorcellen en op bepaalde ziekteverwekkers, zoals de parasiet *Schistosoma mansoni* (Hoofdstuk 3).
In onze zoektocht naar eiwitten en suikerstructuren binnen het menselijk lichaam, die door MGL herkend kunnen worden, vonden wij het CD45 eiwit. Het CD45 eiwit regelt de activiteit van T-cellen. MGL binding aan CD45 vermindert de functie van CD45 en daarmee dus ook de activiteit van de T-cellen. Onder bijzondere omstandigheden kan MGL deze T-cellen zelfs doden (Hoofdstuk 4). MGL controleert vooral de functie van effector T-cellen, een categorie T-cellen die verantwoordelijk zijn voor de daadwerkelijk opruiming van ziekteverwekkers of tumorcellen. Omdat deze effector T-cellen zo efficiënt zijn in het doden van geïnfecteerde of tumorcellen, moeten deze cellen zeer goed in de gaten gehouden worden, opdat zij niet ook allerlei normale weefsels gaan vernietigen. Via de MGL-CD45 interactie kunnen dus tolerogene dendritische cellen de activiteit van effector T-cellen regelen en er voor zorgen dat deze cellen alleen in actie komen op het moment dat het daadwerkelijk nodig is. Een andere bindingspartner van MGL bevindt zich op speciale cellen die de lymfevaten in de lymfeklieren en de zwezerik omgeven, de zogenaamde sinus-endotheelcellen en de lymfe-endotheelcellen (Hoofdstuk 5). Deze interactie zorgt er waarschijnlijk voor dat dendritische cellen, die het MGL molecuul op hun oppervlak dragen op hun plaats blijven en lokaal hun functie kunnen blijven uitoefenen.
Dendritische cellen zijn professionele antigeen presenterende cellen, d.w.z. ze zijn in staat allerlei (lichaamsvreemde) eiwitten of andere substanties (antigenen), uit hun omgeving op te nemen, af te breken en te tonen aan T-cellen. Ook antigenen die rechtstreeks aan MGL binden worden opgenomen in de dendritische cel en gepresenteerd aan het immuunsysteem. Deze opname of internalisatie wordt verzorgd door een speciaal eiwitmotief in de cytoplasmatische staart van het MGL

molecuul (Hoofdstuk 6).
Zoals eerder vermeld bevatten tumoren andere suikers dan normale cellen. Normale cellen hebben meestal lange suikerstructuren (meer dan 20 bouwstenen), terwijl in tumorcellen deze ketens heel kort zijn en maar uit 1 of 2 suikerbouwstenen bestaan. Een voorbeeld hiervan is het MUC1 eiwit. Dit MUC1 komt voor in bijvoorbeeld dikke darmtumoren, maar ook op normale cellen zoals geactiveerde T cellen. Het MUC1 uit tumoren heeft echter een heel ander uiterlijk. Op geactiveerde T cellen bevat het MUC1 eiwit meerdere lange suikerketens, terwijl in darmtumoren er minder ketens met slechts enkelvoudige GalNAc-suikers voorkomen. Juist deze GalNAc-suikers worden efficiënt herkend door het MGL lectine. In ons onderzoek hebben we gevonden dat MGL inderdaad alleen kan binden aan tumor MUC1 en dus selectief onderscheid kan maken tussen de normale cellen en de tumorcellen (Hoofdstuk 7). Mogelijk draagt de interactie tussen MGL op dendritische cellen en MUC1 uit de tumor bij tot de afweerreactie tegen deze tumoren.
Tenslotte hebben we gekeken naar de interacties tussen C-type lectines en ziekteverwekkers en welke gevolgen deze interacties hebben op de functie van dendritische cellen. Twee ziekteverwekkers die specifiek door MGL herkend worden, zijn de bacterie *Campylobacter jejuni* en de parasiet *Schistosoma mansoni*. *Campylobacter jejuni* is de veroorzaker van het Guillain-barré syndroom (een ziekte gekenmerkt door verlammingsverschijnselen). Deze bacteriën hebben de unieke eigenschap dat ze hun eiwitten glycosyleren met onder andere GalNAc-suikers, de bindingsstructuur voor MGL (Hoofdstuk 8). Zo ontdekten wij dat de parasiet *Schistosoma mansoni* naast MGL nog twee andere C-type lectines op het celoppervlak van de dendritische cel bindt, namelijk de mannose receptor en het DC-SIGN lectine. Bepaalde oplosbare antigenen van *Schistosoma mansoni* worden via deze receptoren opgenomen door de dendritische cel en mogelijk ook aan het immuunsysteem gepresenteerd. T-helper cellen zijn T-cellen, die als functie hebben de cellulaire (de T-helper 1 respons) of humorale immuniteit (de T-helper 2 respons) te regelen. *Schistosoma mansoni* binding aan MGL, mannose receptor en DC-SIGN stuurt de dendritische cel aan tot de instructie van T-helper 2 cellen (Hoofdstuk 9). Binding van een *Neisseria meningitidis* mutant (de veroorzaker van bacteriële hersenvliesontsteking) aan DC-SIGN leidt tot de vorming T-helper 1 cellen, waarbij de herkenning van ongemuteerde bacterien juist tot een gemengde T-helper 1/T-helper 2 respons leidt (Hoofdstuk 10).
Een belangrijke conclusie uit ons onderzoek is dat via de interactie van tumoreiwitten of ziekteverwekkers aan C-type lectines de functionaliteit van dendritische cellen en op die manier de hele afweerreactie beïnvloed kan worden. Deze kennis leidt mogelijk in de toekomst tot de ontwikkeling van efficiëntere medicijnen in de bestrijding van infectieziekten, autoimmuunziekten en kanker.

SUMMARY

Our immune system is continuously attacked by foreign pathogens or by cells that have the potential to develop into tumor cells. One major difference between these pathogens, potential tumor cells and our own healthy cells are the glycan structures that are exposed on the cell surface. The sentinels of our immune system, the dendritic cells, have developed special receptors to check upon carbohydrate changes and to initiate an immune response if necessary.

The glycosylation process

For many years, scientists believed that the glycan structures on cell surface glycoproteins were merely decorations. Recent evidence indicates however, that glycans perform very specific roles within the immune system. They are essential for the communication between different leukocyte populations, they regulate the trafficking of cells in the lymph node and the spleen and they aid the immune system in self-nonself discrimination.

How are glycan structures assembled? Newly synthesized proteins are covered with sugars in certain intracellular organelles, such as the endoplasmic reticulum (ER) and the Golgi apparatus. 1-3% of our genes encode for enzymes that are involved in this process of glycosylation. N-linked and O-linked glycosylation are the two most common forms of protein glycosylation. N-linked glycosylation starts by the addition of a preformed glycan chain composed of 9 monosaccharides to the amino acid asparagine. O-linked glycosylation is initiated by the addition of a single N-acetylgalactosamine (GalNAc) to the amino acids serine or threonine. All N- or O-linked glycans are further revised in the Golgi apparatus by enzymes that either remove (glycosidases) or add (glycosyltransferases) monosaccharides to the glycan chain.

The 10 monosaccharides used in mammalian glycosylation can create an enormous diversity of glycans. They can be attached to each other at multiple sites, whereby not only linear but also branched structures are formed. Furthermore, the coupling of 2 monosaccharides can be directed upwards or downwards, leading to millions of possible combinations. For comparison: the 4 bases of the 'DNA-alphabet' can be attached to each other in 256 different ways. With 20 amino acids 160.000 different peptides can be assembled. However, with only 10 simple monosaccharides more than 15 million combinations can be generated.

Every cell, in every tissue or organ, possesses its own composition of the glycosylation machinery, which changes under the influence of the activation status of a particular cell. Alterations occur by aging of the cells, invasion of pathogens or by changes in the local environment of the cell, e.g. through hormonal fluctuations. A tumor cell will therefore display another set of glycans on its cell surface than the parental cell from which the tumor originated. Aberrant glycan structures are found in a wide variety of diseases, including rheumatoid arthritis and some muscle

diseases, where they significantly contribute to disease progression. It is therefore essential to strictly control and regulate the entire glycosylation process. One important task of our immune system is to check for the occurrence of faulty glycan structures.

The immune system

One can consider the immune system to be an army that has to defend its country (the human body) against an enemy (pathogens or tumors). Dendritic cells and T cells are two important leukocyte subpopulations within the immune system. Dendritic cells, named after their many cellular extensions or dendrites (*dendron* is the Greek word for tree), act as sentinels. They are located in all tissues, however they are most abundantly found in those tissues that are in direct contact with the outside world, such as the skin and the mucosal tissues. Our body is continuously attacked by foreign invaders, like viruses and bacteria, or by aberrant tumor cells. Whenever dendritic cells detect such a potential enemy, they become activated and pass the necessary information on to T cells, the soldiers of the immune system. This transfer of information between dendritic cells and T cells occurs in the lymph nodes. Subsequently, the T cells become activated and they head out to counterattack and eliminate the threat.

C-type lectins

Dendritic cells possess several receptors that recognize glycans. Proteins that specifically interact with sugar moieties are referred to as lectins. The large lectin family comprises of several protein families that differ from each other in structure and recognition mode. C-type lectins need a calcium-ion to bind sugars (hence the C). They are transmembrane molecules that consist of a short intracellular (or cytoplasmic) tail, a transmembrane domain and a rigid stalk to which the carbohydrate recognition domain is attached. This carbohydrate recognition domain mediates sugar binding. Lectins are present on many different cell types, however especially dendritic cells express abundant levels of C-type lectins on their cell surface. Comparable to the immunological processes regulated by glycan structures, C-type lectins contribute to cell-cell communication, the trafficking of leukocytes throughout the body and they participate in the recognition of invading pathogens.

This thesis

This thesis, entitled "Novel insights into MGL-glycan interactions in the immune system", describes the results of a study examining the function of the C-type lectins MGL and DC-SIGN. Both these C-type lectins are abundantly expressed on dendritic cells. At the start of this project the field of glyco-immunology was relatively new, so little was known about the C-type lectin MGL. At that time the carbohydrate recognition profile of MGL had not been elucidated. To study MGL function, we generated monoclonal antibodies to MGL, so that we would be able to detect MGL protein expression on isolated cells as well as in human tissues. Using these

antibodies we found that MGL is expressed at elevated levels on a specialized tolerogenic dendritic cell subset, which does not contribute to the initiation of immune responses, but rather inhibits immunity (Chapter 2). Furthermore, we determined the carbohydrate specificity of MGL via glycan microarray analysis. The glycan microarray consists of a small glass slide or plate to which hundreds of different carbohydrate structures have been applied. We added the MGL lectin to the array and examined which sugar moieties specifically interacted with the MGL molecule. By this approach we observed that all MGL-binding glycans contained a terminal GalNAc residue. Terminal GalNAc structures are relatively rare in the human body, however they are abundantly expressed on tumor cells and on certain pathogens, such as the parasite *Schistosoma mansoni* (Chapter 3).

In our search for proteins and carbohydrate structures within the human body that are recognized by MGL, we discovered the CD45 protein. The CD45 protein regulates T cell activity. MGL binding to CD45 blocks CD45 function and thus reduces T cell activity. Under specialized circumstances, MGL binding to CD45 can even kill T cells (Chapter 4). MGL mainly restrains the activity of effector T cells, an important T cell subset in the eradication of tumor cells and pathogens. Although effector T cells are very efficient in eliminating infected cells or tumor cells, they need to be kept under tight control, to prevent destruction of healthy tissues. Via the MGL-CD45 interaction tolerogenic dendritic cells can control effector T cell activity, thereby ensuring that effector T cells only act when appropriate. Another counter-receptor for MGL is found on sinusoidal endothelial cells and lymphatic endothelial cells that line the lymph vessels of the lymph nodes and the thymus respectively (Chapter 5). The interaction between MGL and the sinusoidal and lymphatic endothelial cells probably ensures dendritic cell retention at these sites, allowing the dendritic cells to locally perform their function.

Dendritic cells are professional antigen presenting cells, capable of picking up and degrading foreign proteins or substances (antigens) for presentation to T cells. Also antigens that bind directly to MGL are taken up by dendritic cells and presented to the immune system. Uptake or internalization by MGL depends on a special protein motif in the cytoplasmic tail of MGL (Chapter 6).

As mentioned before, tumor cells display different carbohydrate structures on their cell surface than normal healthy cells. Normal cells usually possess long glycan chains (more than 20 monosaccharides), while in tumor cells these glycans are reduced to 1 or 2 monosaccharides. One example of such a tumor glycoprotein is MUC1. MUC1 is expressed in colon tumors, but also on normal cells, like activated T cells. However, MUC1 from tumors has a different appearance compared to MUC1 from normal cells. On activated T cells MUC1 displays multiple, elongated carbohydrate structures, whereas in colon tumors MUC1 contains less glycans that are composed of single GalNAc residues. These single GalNAc moieties are efficiently recognized by the MGL lectin. We found that MGL can only bind tumor-derived MUC1 and thus distinguish between healthy and tumor tissue (Chapter 7). Therefore, the interaction of MGL and tumor-derived MUC1 might contribute to the

immune response to colon tumors.
Finally, we examined the binding of pathogens to C-type lectins and the functional consequences of such interactions on dendritic cells. Two pathogens that are specifically recognized by MGL are the bacterium *Campylobacter jejuni* and the parasite *Schistosoma mansoni*. *Campylobacter jejuni* infection can cause Guillain-Barré syndrome (a disease characterized by paralysis symptoms). These bacteria have the unique capacity to glycosylate their proteins with terminal GalNAc structures, the determinant for MGL binding (Chapter 8). The parasite *Schistosoma mansoni* not only binds MGL, but is also recognized by two other C-type lectins on the dendritic cell surface, namely the mannose receptor and DC-SIGN. Certain soluble antigens of this parasite are taken up by these receptors and possibly presented to the immune system as well. T helper cells are specialized T cells that aid the formation of a cellular (T helper 1 response) or humoral (T helper 2 response) immune response. *Schistosoma mansoni* binding to the C-type lectins MGL, DC-SIGN and the mannose receptor licenses dendritic cells to instruct a T helper 2 response (Chapter 9). Binding of a mutant form of *Neisseria meningitidis* (the causative agent of bacterial meningitis) to DC-SIGN results in the formation of a T helper 1 response, whereas wildtype bacteria instruct a mixed T helper 1/T helper 2 response (Chapter 10).
One of the main conclusions from our research states that the interaction between tumor glycoproteins or pathogens and C-type lectins influences the functionality of dendritic cells and thus the nature of the immune response. This knowledge might in the future lead to the development of a more efficient treatment in the battle against infections, autoimmune diseases and cancer.

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Het zit erop: 4 jaar promotieonderzoek. Hier is eindelijk dat felbegeerde boekje. Ik had dit nooit succesvol kunnen afronden zonder hulp, dus wil ik hier van de gelegenheid gebruik maken om een aantal mensen persoonlijk te bedanken.
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Sandra

CURRICULUM VITAE

Sandra Johanna van Vliet werd geboren op 31 juli 1971 te Utrecht. In 1989 behaalde zij haar VWO-diploma aan het Niels Stensen College. In hetzelfde jaar begon zij haar HLO-studie aan de Hogeschool Utrecht, waar ze in 1993 afstudeerde als ingenieur in de chemische richting met als specialisatie biochemie. Haar stage bracht zij door in Beerse, België bij Janssen Pharmaceutica op de afdeling Biochemie II van Didier de Chaffoy, waar ze werkte aan de regulatie van apolipoproteïne B synthese in de lever. Vervolgens begon zij als analist bij Carl Figdor en Yvette van Kooyk aan het Nederlands Kanker Instituut, waar ze onderzoek deed naar de ins-en-outs van integrin signalering. Eén jaar later volgde zij Carl Figdor naar het Universitair Medisch Centrum st. Radboud te Nijmegen en 7 jaar later Yvette van Kooyk, toen zij haar eigen onderzoeksgroep startte op de afdeling Moleculaire Celbiologie en Immunologie van het VU Medisch Centrum te Amsterdam. In 2002 volgde een nieuwe uitdaging en begon zij aan een promotieonderzoek naar de regulatie en functie van C-type lectines in dendritische cellen. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Momenteel is zij werkzaam als postdoc in de onderzoeksgroep van Yvette van Kooyk op een project waarin wordt onderzocht hoe de functionaliteit van dendritische cellen wordt beïnvloed door bacteriële oligo-sacchárides.

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1. **van Liempt, E., S. J. van Vliet, A. Engering, J. J. García Vallejo, C. M. Bank, M. Sanchez-Hernandez, Y. van Kooyk and I. van Die**. 2007. *Schistosoma mansoni* soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. Mol Immunol. *In press*
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